The role of Pex3p in Early Events of Peroxisome Biogenesis in *Hansenula polymorpha*

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The role of Pex3p in Early Events of Peroxisome Biogenesis in *Hansenula polymorpha*

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Voorwoord

Het zit erop, `t is af, bijna klaar...
Straks nog de promotie, en dan kan ik mijn AIO-schap in Haren definitief tot het verleden rekenen. Het voelt ook echt een beetje als “verleden” omdat ik inmiddels alweer een hele tijd in Amsterdam als post doc aan de slag ben. Al met al heeft het bereiken van dit moment wat langer geduurd dan ik zelf had gehoopt, maar het is dan toch zover. Rest mij nog diegenen te bedanken, die een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift.
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Ik hou van je.

Gert Jan

Voor mijn ouders

Contents

Chapter 1

General Introduction

Abstract

Peroxisomes are versatile organelles that fulfill important functions in eukaryotic organisms, ranging from yeast to man. In the recent past, much progress has been made in unraveling the molecular mechanisms involved in the biogenesis of this organelle. 23 different genes (PEX genes) have been identified that are involved in peroxisome biogenesis. This chapter gives a general description of peroxisome function, structure, and biogenesis. Emphasis is given to the possibility that peroxisomes are formed de novo.

Introduction

Eukaryotic cells are subdivided into distinct cellular compartments, designated organelles. Microbodies (peroxisomes, glycosomes, glyoxysomes, and hydrogenosomes) represent the most recently discovered class of organelles.

Peroxisomes are devoid of DNA, and all peroxisomal proteins studied thus far are synthesized on free cytosolic polysomes (Lazarow and Fujiki, 1985). This implies that proteins, which are destined for the peroxisomal matrix or membrane, need to be imported into the organelle post-translationally via specific import machineries. The details of the mechanism by which import occurs are currently emerging from studies in different model organisms, ranging from yeast to man, performed in various research groups.

Peroxisomes represent a highly dynamic component of the cellular machinery, and the number and size of these organelles is strongly determined by cell type and physiology. Peroxisome homeostasis not only requires careful regulation of organelle biogenesis and function, but also organelle numbers. Recently, several research groups have devoted their efforts at unraveling the mechanism of peroxisome degradation.

This general introduction summarizes our current understanding of the mechanisms of peroxisome biogenesis. It will focus on the possibility that peroxisomes are derived from non-peroxisomal cellular membranes. Recent data indicate that this possibility might exist.

Peroxisome structure and function

Microbodies represent the class of most recently discovered organelles (for a review see Purdue and Lazarow, 2001). They were first observed in 1954 by Rhodin (Rhodin, 1954) and their generalized name referred to their small size, which made

them overlooked for such a long period of time. Several years later microbodies were further classified on the basis of their metabolic function and discriminated in peroxisomes, glycosomes, glyoxysomes, and hydrogenosomes. Peroxisomes range in size from 0.1 to 1 μm and are bounded by a single membrane. They can assume different shapes depending on the type of cell that is studied. They can be spherical, tubular, or even almost cubic (in methylotrophic yeasts grown on methanol), and sometimes are interconnected ("peroxisomal reticulum"). The peroxisomal membrane is the boundary between the cytosol and the protein-rich peroxisomal matrix. Although the term microbody is still used by some scientists to describe the morphological appearance of these organelles, the name peroxisome was given to a subclass of these subcellular compartments in 1966 by de Duve and Baudhuin (De Duve and Baudhuin, 1966), after biochemical analysis of their metabolic function. They established that peroxisomes are the cellular compartment where most hydrogen peroxide metabolism takes place, hence their name. Another major and well-conserved function of peroxisomes is fatty acid β-oxidation. Microbody function is often highly specified by organism and cell type and can include such diverse processes as cholesterol and ether lipid (plasmalogen) synthesis in animals, glycolysis in trypanosomes (glycosomes), photorespiration in leaves, and glyoxylate metabolism in germinating seeds (glyoxysomes) (for review, see Purdue and Lazarow, 2001). In yeasts, peroxisomes are involved in the primary metabolism of specific carbon and nitrogen sources (Veenhuis and Harder, 1988). In methylotrophic yeast species (such as *Hansenula polymorpha* and *Pichia pastoris*) the degradation of methanol and primary amines is confined to these organelles. (For reviews of peroxisome biochemistry, see: Veenhuis and Harder, 1988; van den Bosch et al., 1992.) The enzyme activities catalyzing the above processes are located in the peroxisomal matrix (lumen). In *Neurospora crassa* the Woronin body was recently identified as a specialized peroxisome-derived organelle (Jedd and Chua, 2000). In this filamentous fungus, these organelles function as plugs for septal pores to avoid cytoplasmic bleeding caused by mechanical damage. The importance of peroxisomes is further demonstrated by the existence of several diseases in man associated with peroxisome malfunction, the Peroxisomal Biogenesis Disorders (PBDs), some of which are lethal (reviewed in Gould and Valle (2000)).

Peroxisome biogenesis is governed by the activities of a distinct class of proteins termed peroxins (Distel et al., 1996). These peroxins are encoded by *PEX* genes. Their function in peroxisome biogenesis and performance is detailed below.

Peroxisome proliferation

In yeasts, several growth substrates, including alkanes, D-amino acids, oleic acid and methanol induce peroxisome biogenesis (reviewed in Veenhuis and Harder (1988)). Growth conditions, such as type of substrate used, growth rate, and oxygen availability determine the number and size of the organelles, not the amount of peroxisomal proteins synthesized (Veenhuis and Harder, 1988). An example of this is observed in *H. polymorpha pim* mutants, in which a major portion of peroxisomal matrix proteins is mislocalized to the cytosol. In these mutants, small peroxisomes may be present at numbers comparable to wild type cells.

An important characteristic of peroxisomes in *H. polymorpha* is that they are capable of importing matrix proteins during a limited period in their existence. Small peroxisomes grow due to incorporation of lipids and proteins; after their maturation one or a few new organelles form by fission. The mature organelle ceases to grow, while the newly formed peroxisome increases in size. This new organelle apparantly has “inherited” the ability to import new components, leaving the mature organelle as an import-incompetent “enzyme bag” (Veenhuis et al., 1989; Waterham et al., 1992).

The factors involved in regulation of peroxisome proliferation and maturation are largely unknown. However, the peroxisomal membrane protein Pex11p seems to play a role in peroxisome multiplication. Overproduction of Pex11p leads to proliferation of peroxisomes, while giant peroxisomes are found in a *pex11* deletion strain (Erdmann and Blobel, 1995; Marshall et al., 1995). Marshall et al. (1996) suggested that the oligomeric state of Pex11p could be instrumental in peroxisome maturation. They found monomeric Pex11p associated with small, immature organelles, while dimeric protein was present in mature ones. The exact function of Pex11p in peroxisome maturation is clearly not resolved yet. In a more recent study, a direct role of Pex11p in transport of medium chain fatty acids into peroxisomes of *S. cerevisiae* was suggested by van Roermund et al. (2000). In their view, the proliferation of organelles is caused as an effect of increased β-oxidation. However, Gould and co-workers challenged this view and showed that this could not be the only reason. They observed a direct effect of Pex11p levels on peroxisome numbers, even in the absence of peroxisomal metabolic activity (Li and Gould, 2002). Further research is needed to unravel the principles of peroxisome maturation and proliferation.

After peroxisomes have become redundant for growth, e.g. after a shift of cells from peroxisome-inducing to peroxisome-repressing conditions, the organelles are rapidly degraded by a selective process called pexophagy (reviewed by Bellu and Kiel). In

H. polymorpha, this process mainly targets the mature organelles, leaving the small, import-competent peroxisomes unaffected. Recently, it was shown that a protein involved in biogenesis of peroxisomes, Pex14p, also plays a role in pexophagy (Bellu et al., 2001). Pex14p might function as a molecular switch discriminating between import competence and incompetence of peroxisomes, enabling cells to swiflty adapt to new growth conditions that require new peroxisomal metabolic activities.

Isolation of *pex* mutants and cloning of *PEX* genes

Yeasts are excellent model organisms for studies of the mechanisms of peroxisome biogenesis by virtue of the fact that mutants that are affected in this process, the so-called *pex* mutants, are viable. Yeast *pex* mutants are unable to grow on specific media that require peroxisome function (e.g. oleate or methanol) enabling easy selection. However, normal growth can occur when non-selective substrates (e.g. glucose) are used. Complementation of the *pex*-specific growth defect leads to the isolation of the corresponding *PEX* gene. Over the past two decades 23 peroxins have been identified (see Table 1, and http://www.peroxisome.org/). For many of these, both the yeast and mammalian genes have been cloned and sequenced. Many of the *pex* mutants in different organisms contain peroxisomal membrane remnants, or ghosts (Santos et al., 1988; Santos et al., 2000). This is indicative of the fact that although import of peroxisomal matrix proteins is blocked in these mutants, they are still able to assemble and maintain their membrane, including (a portion of) their proteins. An increasing amount of data is accumulating contributing to our knowledge of peroxisome biogenesis, e.g. how peroxisomal matrix proteins are targeted to their subcellular localization.

Function and interactions of peroxins

Matrix protein import

A number of the peroxins identified thus far, is involved in matrix protein import. The corresponding *pex* mutants contain ghosts (see above) and mislocalize peroxisomal matrix proteins to the cytosol. This clearly sets this type of mutant apart from another class, which seems to lack any detectable form of peroxisomal remnants in some organisms. These mutants (*pex3*, *16*, *17*, and *19*) are affected in the biogenesis and maintenance of the peroxisomal membrane itself.

Most matrix proteins are targeted to the peroxisomal matrix by the Peroxisomal Targeting Signal (PTS) 1, consisting of a C-terminal tripeptide -SKL and its variants

Table I. Overview of peroxins

Peroxin	PBD CG?	Protein interactions	Notes
PEX1	1	PEX6	AAA ATPase required for matrix protein import
PEX2	10		C3CH4 zinc-binding integral PMP required for matrix protein import
PEX3	12	PEX19	Integral PMP required for membrane biogenesis
PEX4	n.d.	PEX22	E2 ubiquitin-conjugating enzyme required for matrix protein import
PEX5	2	PEX7, PEX14, PEX13, PEX10, PEX12, the PTS1	TPR-containing PTS1 receptor, required for PTS1-mediated import (yeast) or both PTS1- and PTS2-mediated import (human)
PEX6	4	PEX1	AAA ATPase required for matrix protein import
PEX7	11	PEX5, PEX14, PEX13, the PTS2	WD-40 repeat containing PTS2 receptor required for PTS2-mediated import
PEX8	n.d.	PEX5	Integral PMP required for matrix protein import, contains a PTS1 as well as a PTS2
PEX9	n.d.	n.d.	Integral PMP required for matrix protein import
PEX10	7	PEX12, PEX5, PEX19	C3CH4 zinc-binding integral PMP required for matrix protein import
PEX11	n.d.	PEX19	PMP involved in peroxisome proliferation
PEX12	3	PEX10, PEX5, PEX19	C3CH4 zinc-binding integral PMP required for matrix protein import
PEX13	13	PEX5, PEX7, PEX14	SH3 containing PMP required for matrix protein import
PEX14	14	PEX5, PEX7, PEX13, PEX17	PMP required for matrix protein import, the initial site of receptor docking
PEX15	n.d.	n.d.	PMP required for matrix protein import
PEX16	9	PEX19	Integral PMP required for membrane biogenesis
PEX17	n.d.	PEX14	PMP required for matrix protein import
PEX18	n.d.	PEX7	Required for PTS2-mediated import, thought to be involved in peroxisomal localization of PEX7, highly homologous to PEX21
PEX19	14	Multiple PMPs	Predominantly cytosolic, partly peroxisomal protein involved in peroxisomal membrane protein import
PEX20	n.d.	Thiolase	Required for thiolase import in Y. lipolytica
PEX21	n.d.	PEX7	Required for PTS2-mediated import, thought to be involved in peroxisomal localization of PEX7, highly homologous to PEX21
PEX22	n.d.	PEX4	PMP required for matrix protein import, thought to be a docking factor for PEX4
PEX23	n.d.	n.d.	PMP required for matrix protein import
Djp1	n.d.	n.d.	DnaJ-like protein involved in peroxisomal matrix protein import

PBD CG: Peroxisome Biogenesis Disorder Complementation Group

For references, see: http://www.peroxisome.org

(Gould et al., 1989); (Lametschwandtner et al., 1998). A minor subset of matrix proteins contains a PTS2, a nonapeptide with consensus sequence (R/K)-(L/V/I)-X_5-(H/Q)-(L/A) present near the N-termini of these proteins ((Swinkels et al., 1991); for an overview of PTS2 proteins, see (Chudzik et al., 2000)). Both PTS1 and PTS2 proteins are recognized by specific cytosolic receptors, Pex5p and Pex7p, respectively.

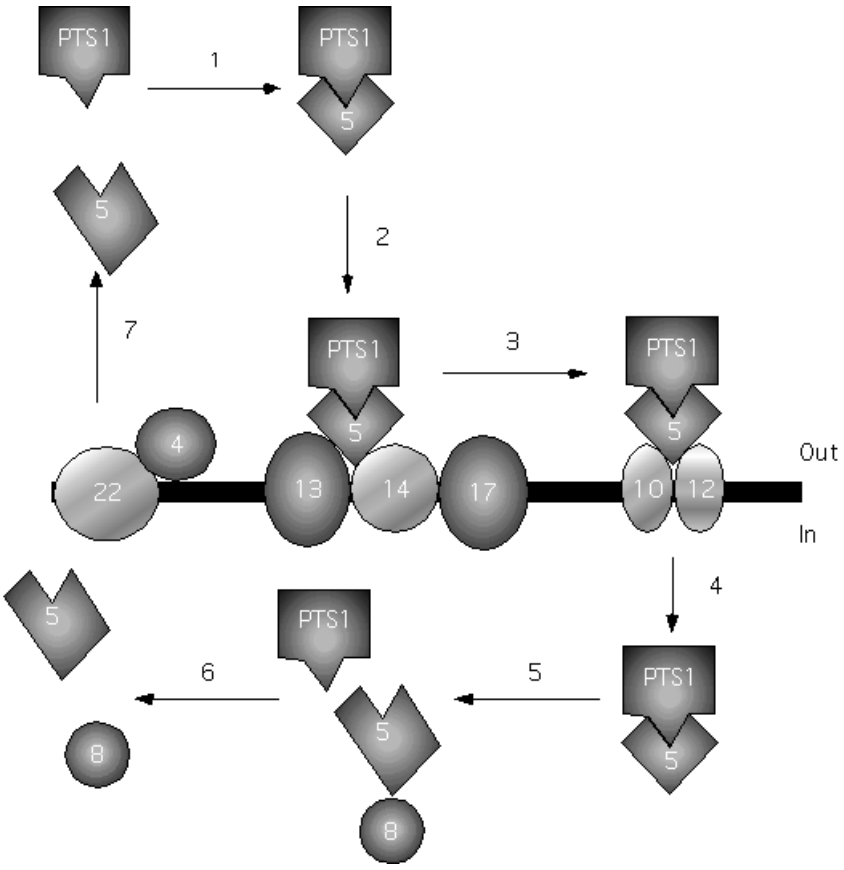

Fig. 1. *Schematic representation of PTS1 matrix protein import in* H. polymorpha*. Several steps can be distinguished in this process, as indicated.* ***(1)*** *In the cytosol the PTS1 receptor Pex5p binds to the PTS1 of newly synthesized matrix proteins.* ***(2)*** *The Pex5p-cargo complex docks to the peroxisomal docking site, which contains Pex13p, Pex14p, and Pex17p.* ***(3)*** *Subsequently, Pex5p interacts with Pex12p, which in turn is in close proximity of Pex10p. Pex10p and Pex12p form the putative entry site (or translocon) for matrix protein import.* ***(4)*** *The Pex5p-cargo complex enters the peroxisome, after which* ***(5)*** *Pex5p releases from its cargo. Pex8p might aid this process, and is subsequently released in the peroxisomal matrix* ***(6)****. Finally,* ***(7)*** *Pex5p is recycled to the cytosol. This process involves Pex4p, which is anchored to the membrane by Pex22p. (Adapted from Veenhuis et al, 2000)*

Pex5p (McCollum et al., 1993) and Pex7p (Marzioch et al., 1994) have been identified in several organisms. In a *pex5* mutant the import of PTS1-containing peroxisomal matrix proteins is disturbed, while in a *pex7* mutant PTS2 proteins are mislocalized to the cytosol. In man, Pex5p and Pex7p have been shown to interact

directly. This interaction is regulated at the level of alternate splicing. Human Pex5p exists in two forms, a long (Pex5pL) and a short (Pex5pS) form, differing by the presence of a 37-amino acid internal region encoded by exon 8 of the *PEX5* gene. This particular region is responsible for the interaction of Pex5p with Pex7p (Braverman et al., 1998). In *Yarrowia lipolytica* and *Caenorhabditis elegans*, no evidence for the presence of the *PEX7* gene has been found thus far. Instead, Pex20p was identified as a cytosolic factor involved in thiolase import (Titorenko et al., 1998). Pex20p plays a role in dimerization and transport of thiolase, and was shown to interact with Pex8p (Smith and Rachubinski, 2001).

The interaction of Pex5p with PTS1-containing proteins has been studied in detail. Several groups have shown independently, that the tetratricopeptide repeats (TPR) present in the C-terminal two-thirds of Pex5p, bind the PTS1 (Brocard et al., 1994; Fransen et al., 1995; Terlecky et al., 1995; Szilard and Rachubinski, 2000). Detailed information on binding of PTS1 by the TPR domains came from the 3-dimensional structure analysis of the C-terminus of human Pex5p (Gatto, Jr. et al., 2000), and from a mutational analysis of *S. cerevisiae* Pex5p (Klein et al., 2001) The localization of both receptors has been a matter of debate for some time. In different studies performed in several species, both proteins have been found in the cytosol, in the peroxisomal matrix, or in both (Dodt et al., 1995; van der Klei et al., 1995; Szilard et al., 1995; Zhang and Lazarow, 1995; Elgersma et al., 1998). A dual localization of the receptors could indicate that import of receptor-cargo complexes takes place via the so-called ‘shuttling-receptor’ model. In this model, the receptor protein, after binding its cargo molecule, docks to a site on the peroxisomal surface and is then translocated across the membrane into the organellar matrix. After translocation, the cargo is released from the receptor and the latter is transported back into the cytosol. Recently, experimental evidence was obtained in the group of Subramani, which indicates that this model is true for human organelles (Dammai and Subramani, 2001). Whether Pex7p behaves in a similar fashion remains a matter of debate.

A third type of PTS (sometimes referred to as PTS3) was found in acyl-CoA oxidase of *S. cerevisiae* and *Candida tropicalis* (Small et al., 1988). *S. cerevisiae* catalase A seems to contain both a PTS1, as well as an internal PTS3. Using the yeast two-hybrid system as well as *in vitro* binding studies, Skoneczny and Lazarow found an interaction between *S. cerevisiae* acyl-CoA oxidase and Pex5p, the PTS1 receptor, suggesting a role for the PTS1 receptor in PTS3 import (Skoneczny and Lazarow, 1998).

The factors involved in recycling of Pex5p remain unknown thus far. However, in *H. polymorpha*, Pex4p seems to play a direct role in recycling of Pex5p. Overexpression of *PEX5* in a *pex4* strain leads to the accumulation of Pex5p in the peroxisomal matrix (van der Klei et al., 1998). Pex4p is a member of the E2 family of ubiquitin-conjugating enzymes (Wiebel and Kunau, 1992). This protein is anchored to the peroxisomal membrane by Pex22p, an integral membrane protein (Koller et al., 1999). Both Pex4p and Pex22p are essential for PTS1 and PTS2 import; in *H. polymorpha* however, Pex4p is dispensable for PTS2 import (van der Klei et al., 1998). The target molecule for ubiquitination by Pex4p is unknown, although it was hypothesized that Pex18p and Pex21p might be the targets in *S. cerevisiae*. A double knock-out *Δpex18*/*Δpex21* strain of baker's yeast shows an import defect of PTS2 proteins, whereas PTS1 import occurs normally (Purdue et al., 1998). A single deletion of either of the genes only has a small impact on PTS2 import. Both proteins are very unstable *in vivo* (half-life of <10 min; (Purdue and Lazarow, 2001)), and Pex18p becomes mono- and di-ubiquinated during peroxisome biogenesis. Possibly, this ubiquitination of Pex18p represents the link between Pex4p activity and receptor recycling (see above). However, it should be noted that *H. polymorpha* Pex4p is exclusively involved in PTS1 import (van der Klei et al., 1998).

After binding of proteins destined for the peroxisomal matrix to their respective receptors, the next step in the sequence of events leading to import is binding of the receptor-cargo complex to a docking site localized on the cytosolic face of the peroxisomal membrane. This role is fulfilled by Pex13p (Gould et al., 1996), Pex14p (Gould et al., 1996; Komori et al., 1997; Will et al., 1999), and Pex17p (Huhse et al., 1998). Pex13p and Pex14p were identified as binding partners for both Pex5p and Pex7p in multiple studies (Elgersma et al., 1996); (Girzalsky et al., 1999); (Fransen et al., 1998); (Shimizu et al., 1999). Since both Pex5p and Pex7p bind to Pex13p and Pex14p, the docking complex is actually the site were the PTS1 and PTS2 pathways converge. This also explains why mutations in peroxins acting downstream of Pex13p/Pex14p, typically affect both import routes. Although both proteins appear to play a role in import of matrix proteins, the *pex14* phenotype can be rescued by overexpression of Pex5p in *Hansenula polymorpha*, indicating that Pex14p's function can be circumvented in this organism (Salomons et al., 2000). Possibly, Pex14p is only required for enhancing the efficiency of import, conceivably by directing receptor-cargo complex towards the docking site (Pex13p).

After binding of the receptor-cargo complex to the putative docking site on the cytosolic face of the peroxisomal membrane, translocation of PTS-containing matrix

proteins across the organellar membrane must take place. Little is known about the mechanism of translocation. It is tempting to speculate that the three ring-finger containing proteins, Pex2p (Tsukamoto et al., 1991), Pex10p, and Pex12p (Chang et al., 1999; Okumoto et al., 2000) (all integral components of the peroxisomal membrane) are involved in this step. The significance of the ring-finger domains for their function is not clear, however, mutational analysis of this domain indicates that it is essential for proper functioning of Pex2p in matrix protein import, but not for Pex5p docking. It has been suggested that Pex2, 10 and 12p might form the core translocon that accommodates the transfer of receptor-cargo complexes across the membrane. Such a putative peroxisomal translocon needs to meet at least two important prerequisites: First, translocation of proteins must be mediated without disrupting the solute gradients that exist across the membrane. Secondly, the translocon must be able to accommodate very large structures. It is a well-established fact that peroxisomes maintain a pH gradient (Nicolay et al., 1987; Dansen et al., 2000), suggesting that even molecules as small as protons are not able to passively traverse the translocation apparatus. This suggests that a mechanism for preventing leakage of small molecules must exist. The first possible mechanism requires that the translocation pore is effectively sealed during its activities, either by means of an external “plug” (comparable to the function of the lumenal ER-chaperone Kar2p (BiP) in transport of secretory proteins into the ER lumen (Haigh and Johnson, 2002)), or by a tightly regulated concerted closing and opening of the putative translocon itself. In another conceivable mechanism, import of matrix proteins does not occur via a translocon, but rather by a vesicle fusion event. The second prerequisite for the peroxisomal translocon (the ability to accommodate large structures) is most dramatically illustrated by the work of Walton and co-workers, who showed that gold particles, ranging in size from 4-9 nm, coated with PTS1-containing proteins and microinjected into cultured mammalian cells, could be taken up by peroxisomes (Walton et al., 1995). Other examples of import of quite large molecules into peroxisomes exist, e.g. the import of tetrameric catalase (~ 240 kDa (Brul et al., 1988)).

Two proteins belonging to the family of AAA-ATPases, Pex1p and Pex6p, are involved in peroxisome biogenesis (Erdmann et al., 1991; Faber et al., 1998). One of the many cellular functions in which AAA-ATPases play a role is membrane fusion (mammalian NSF and *S. cerevisiae* Sec18p (Malhotra et al., 1988; Eakle et al., 1988)). Interestingly, Pex1p and Pex6p have been localized both to vesicles as well

as to peroxisomes. This suggests that a fusion event might occur during some stage of peroxisome biogenesis, possibly involving vesicles.

Even though we posses some information on the characteristics of the peroxisomal translocon, its nature still eludes us. When studied by electron microscopy, and more in particular freeze etch techniques, there is no sign of the presence of large protein complexes in the peroxisomal membrane (as one would expect if a large pore analogous to the nuclear pore complex would be present). Also biochemically, no clear evidence for the existence of such a complex has been found yet, although recent work in our group using blue native gel electrophoresis has provided preliminary evidence for the existence of a peroxisomal membrane protein complex.

Membrane protein insertion

The membrane surrounding peroxisomes contains several proteins, which are either involved in peroxisome biogenesis and maintenance (membrane-bound peroxins), or in transport and enzymatic functions (e.g. PMP70 in humans, or Pat1p, Pat2p, and PMP47 in yeasts).

Compared to our understanding of the targeting of matrix proteins to the peroxisome, little is known about insertion of membrane proteins into the peroxisomal membrane. Determining the minimal targeting information for peroxisomal membrane proteins (the mPTS) has proven to be cumbersome. In general, approaches using mutagenesis of membrane proteins harbour the intrinsic difficulty that great care must be taken to avoid misinterpretations caused by, for instance, instability of mutant proteins. Furthermore, the tendency of membrane proteins to aggregate or randomly insert into membranes after removal of their proper targeting information is cause for concern in these types of studies.

Recently, Gould and co-workers have directed their attention towards a re-evaluation of the mPTS in peroxisomal membrane proteins (PMPs). They identified two regions in human PMP34 that seem to function as mPTSs (Jones et al., 2001). They speculate that the multiple mPTSs in a single PMP might function as binding sites for a specific PMP binding factor that is required for targeting of PMPs to the peroxisomal membrane. PMPs containing more than one membrane-spanning domain (like PMP34) could harbor more than one mPTS.

In vitro studies on insertion of peroxisomal membrane proteins into isolated organelles have resolved some of the minimal requirements of this process. These studies have revealed two distinct steps in membrane insertion: (1) Binding of the protein to the membrane, and (2) correct insertion into the membrane (Diestelkotter

and Just, 1993; Just and Diestelkotter, 1996; Imanaka et al., 1996). Insertion of the model proteins PMP22 and PMP70 did not require ATP or GTP, whereas Pex2p-insertion required the presence of ATP, but not its hydrolysis. Protease pre-treatment of isolated organelles showed the involvement of proteinaceous factors, which are insensitive to *N*-ethylmaleimide. Cytosolic factors are involved in this process (Imanaka et al., 1996; Pause et al., 1997). These cytosolic factors might include molecular chaperones involved in maintaining the hydrophobic membrane proteins in an import-competent state, rather than allowing their aggregation. Pex19p seems to be the cytosolic receptor for peroxisomal membrane proteins. Pex19p was shown to bind to many peroxisomal membrane proteins. Furthermore, targeting of Pex19p to the nucleus by means of a Nuclear Localization Signal (NLS) led to accumulation of PMPs in the nuclear envelope (Fransen et al., 2001; Snyder et al., 2000).

The question of the origin of the peroxisomal membrane is analogous to the general issue of peroxisomal origin. Peroxins (or other types of proteins) directly involved in biosynthesis of the membrane of peroxisomes probably exist, but so far only circumstantial evidence for this hypothesis has been found. In particular Pex3p, is thought to be directly involved in biogenesis of the peroxisomal membrane. This notion is substantiated by the complete lack of peroxisomal remnants in a *pex3* mutant, and by the observation that the integrity of the peroxisomal membrane is affected in a conditional *pex3* mutant, after synthesis of Pex3p is blocked (Baerends et al., 1996).

Models for peroxisome biogenesis: Past and present

The possibility that the ER or nuclear membrane play an active role in peroxisome biogenesis, as already indicated by the early morphological work on microbodies, has been the subject of many recent studies. By now, an impressive amount of evidence has been gathered, pleading the case for an active role of the ER in peroxisome biogenesis.

Titorenko *et al*. discovered the existence of five peroxisomal subpopulations (P1-P5) that consist of pre-peroxisomal vesicles which develop into mature peroxisomes (P6) by a series of fusion events. This process was also reconstituted *in vitro* and its minimal requirements were determined. Strikingly, the same group discovered overlappig phenotypes between mutants affected in the general secretion pathway (*sec238* and *srp54*), and *pex* mutants (*pex1*, *2*, *5*, *6*, *8*, *9*, and *16*). Furthermore, Pex2p and Pex16p of *Y. lipolytica* are N-linked core glycosylated, indicating that these proteins have been in direct contact with the ER lumen.

Overexpression of *PEX* genes often leads to strong proliferation of ER membranes, although Stroobants et al. (Stroobants et al., 1999) propose that this phenomenon is caused by a general stress response.

The observation that ARF and all eight subunits constituting the COPI coatomer complex bind to peroxisomes in rat liver implies the possibility of vesicles budding from the peroxisome. It might be envisaged that this process is important in fission of peroxisomes, especially since Pex11p is able to bind coatomer and ARF (Passreiter et al., 1998). Coatomer and ARF are cellular components involved in vesicle-mediated transport processes. Pex11p contains a dilysine motif, which functions in ER retention of integral membrane proteins. Dilysine motifs facilitate retrieval of ER-resident proteins from ER-Golgi intermediate compartments back to the ER.

In *H. polymorpha* it was shown that BFA, a fungal toxin which blocks vesicle-mediated transport, also affects peroxisome biogenesis and leads to accumulation of both peroxisomal membrane (Pex3p, Pex14p) and matrix (AOX, Pex8p) proteins to the ER (Salomons et al., 1997). Furthermore, targeting studies on Pex3p revealed that the N-terminal 16 amino acids of this protein efficiently target a marker protein to the ER (Baerends et al., 1996). When the Pex3p fragment was extended to 37 residues, the same marker protein was targeted to peroxisomes. The first 50 amino acids of Pex3p drive the formation of ER-derived vesicles in a *pex3* mutant, which can function as precursors for formation of mature peroxisomes after re-introduction of full-length Pex3p (Faber et al., 2002; see chapter 4). Whether this phenomenon indicates a pathway that is significant in WT *H. polymorpha in vivo*, or whether it represents a special rescue mechanism that only occurs in the absence of peroxisomal membrane structures remains to be resolved. Recently, Hazra et al. identified the presence of peroxisomal remnants in a *P. pastoris pex3* knock out strain (Hazra et al., 2002). This indicates that in this organism rescue of peroxisome biogenesis in a *pex3* background need not occur via an alternative route, but rather proceeds by incorporating proteins and lipids into the remnants.

Taken together, there is now much evidence indicating the existence of a pathway other than the “growth and fission” model for biogenesis of peroxisomes. However, the issue is still matter of vigorous debate. New and more conclusive evidence will be needed to decide whether the proposed alternative pathway really plays a role in peroxisome biogenesis *in vivo*.

Aim and outline of this thesis

The objective of this study was to analyse the early events of peroxisome biogenesis in the methylotrophic yeast *Hansenula polymorpha*. This approach was inspired by the observation that specific mutants of this organism completely lack detectable peroxisomal remnants, but nevertheless are able to form new organelles shortly after the specific defect was complemented. The analysis of initial development of these newly formed organelles was expected to further deepen our insight into the cellular origin of peroxisomes.

In order to expand the existing possibilities to introduce gene constructs into *H. polymorpha*, either for industrial or scientific purposes, we set out to develop a new expression system exploiting a *H. polymorpha* auxotrophic mutant. Chapter 2 describes the cloning of two genes, *PUR7* and *PUR8*, and the further exploitation of the *PUR7* gene to construct pHIPA4. It is shown that pHIPA4 is well suited for mild overexpression of genes.

The *pex3* mutant of *H. polymorpha* completely lacks detectable peroxisomal remnants. When the function of the *PEX3* gene is restored, new organelles appear within a short time span. Therefore, we assume that the Pex3 protein might play an important role in the early events of peroxisome biogenesis. We first studied the localization of Pex3p in detail, as described in chapter 3. The results show that *H. polymorpha* Pex3p is not an integral protein as suggested previously. Instead, it is tightly bound to the cytosolic face of the peroxisomal membrane. Furthermore, using GFP as fluorescent marker, distinct focal concentrations of Pex3p.GFP were observed at the peroxisomal membrane, suggesting that Pex3p is present in protein clusters.

In chapter 4 the events occurring after reintroduction of a 50 amino acid long N-terminal fragment of Pex3p fused to GFP into the *pex3* mutant are studied. Surprisingly, large numbers of vesicles accumulate in this situation that contain N_{50}. Pex3p. We show that the vesicles originate from the nuclear membrane and possess a number of peroxisomal characteristics. Also, these vesicles are the sole target of subsequently reintroduced full-length Pex3p and serve as templates for the biogenesis of peroxisomes.

In continuation of this, the research described in chapter 5 provides evidence for the possibility that peroxisomes are formed at the nuclear membrane in *pex3* cells after reintroduction of the complete *PEX3* gene.

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Chapter 2

Characterization of the *Hansenula polymorpha PUR7* gene and its use as selectable marker for targeted chromosomal integration

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Abstract

The *Hansenula polymorpha* genes encoding the putative functional homologs of the enzymes involved in the seventh and eighth step in purine biosynthesis, *HpPUR7* and *HpPUR8*, were cloned and sequenced. An overexpression vector designated pHIPA4 was constructed, that contains the *HpPUR7* gene as selectable marker and allows expression of genes of interest via the strong, inducible alcohol oxidase promoter. An *ade11* auxotrophic mutant that is affected in the activity of the *HpPUR7* gene product was used to construct strain NCYC495 *ade11.1 leu1.1 ura3*. This strain grew on methanol at WT rates (doubling time of approximately 4 h) and is suitable for independent introduction of four expression cassettes, each using one of the markers for selection, in addition to the zeocin resistance marker. It was subsequently used as host for overproduction of two endogenous peroxisomal matrix proteins, amine oxidase and catalase. Efficient site-specific integration of pHIPA4 and overproduction of amine oxidase and catalase is demonstrated. The expression cassette appeared to be pre-eminently suited to mediate moderate protein production levels. The advantages of pHIPA4 and the new triple auxotrophic strain in relation to the use of *H. polymorpha* as a versatile cell factory or as model organism for fundamental studies on the principles of peroxisome homeostasis is discussed.

Introduction

Methylotrophic yeast species like *Pichia pastoris* and *Hansenula polymorpha* are attractive hosts for the production of various proteins of scientific or commercial interest (for review, see van Dijk et al., 2000). The ability of these organisms to grow to high cell densities on relatively inexpensive carbon sources as well as the availability of strong, tightly regulated promoters renders them favorable cell factories. Over the past years, advanced molecular genetic tools have become available for *H. polymorpha*, including an efficient electro-transformation procedure (Faber et al., 1994b), efficient methods for homologous integration into genomic sequences (Faber et al., 1992; Sohn et al., 1996), as well as a method for the rapid selection of integrants with various copy numbers (Agaphonov et al., 1999).

Several selectable markers have been used to enable selection of *H. polymorpha* transformants. Auxotrophic mutants that are available for this purpose include *leu1.1*, *ura3*, and *trp3*. Vectors carrying either homologous (*LEU1*, *URA3*, and *TRP3*) or heterologous (*LEU2* and *URA3* from *Saccharomyces cerevisiae*, *LEU2* from *Candida albicans*) marker genes were constructed that functionally complement these mutations (Agaphonov et al., 1994; Agaphonov et al., 1999). Hollenberg and co-

workers used the *S. cerevisiae URA3* marker that poorly complements the *H. polymorpha ura3* mutation to obtain high copy-number integration of expression cassettes in *H. polymorpha* (Roggenkamp et al., 1986; Janowicz et al., 1991). However, the availability of additional selectable markers is crucial to further increase the potentials of the organism.

The present paper describes the cloning and sequencing of the *H. polymorpha PUR7* gene, encoding phosphoribosyl-aminoimidazole-succinocarboxamide synthetase (SAICAR synthetase; EC 6.3.2.6), and the putative *HpPUR8* gene encoding adenylosuccinate lyase (EC 4.3.2.2). These enzymes perform the seventh and eighth step in the purin biosynthetic pathway, respectively (for review, see Kappock et al., 2000).

The *H. polymorpha ade11.1* (*pur7*) mutant, that is unable to grow in the absence of adenine, accumulates the typical red intracellular pigment, presumed to be 5-aminoimidazole-4-carboxylic acid ribonucleotide, when grown on media containing limiting amounts of adenine (Crowley and Kaback, 1984; Sasnauskas et al., 1991). The red color of this mutant provides an easy additional criterion for distinguishing transformants from non-transformed cells and thus is a useful host strain for genetic manipulation. We describe the construction of a vector carrying the *HpPUR7* gene as selectable marker that cannot replicate in *H. polymorpha*, and the successful application of this integrative vector for overexpression of two homologous genes, encoding *H. polymorpha* amine oxidase (AMO) and catalase (CAT).

Materials and methods

Strains, media and growth conditions

For cloning purposes, *Escherichia coli* DH5α was used and grown as described (Sambrook et al., 1989). *Hansenula polymorpha* strain NCYC495 and auxotrophic derivatives (Gleeson and Sudbery, 1988) were grown at 37°C in YPD medium containing 1% yeast extract, 1% peptone, and 1% glucose, or in synthetic medium (van Dijken et al., 1976). Glucose or methanol was added as a carbon source to a final concentration of 0.5%. For selective growth, cells were plated on YND medium containing 0.67% yeast nitrogen base (Difco, Detroit, MI) and 1% glucose, supplemented with 1.5% agar. When necessary, adenine, leucine, and uracil were added to a final concentration of 30 μg ml $^{-1}$ each.

For biochemical analyses, selected strains were precultured at least three rounds in synthetic medium containing glucose and subsequently shifted to methanol-

containing synthetic medium to induce expression of the gene under the control of the alcohol oxidase promoter. Crossing of auxotrophic mutants of *H. polymorpha* was performed using the procedure described by Gleeson and Sudbery (1988).

Molecular biological techniques

Standard recombinant DNA procedures were carried out as described (Sambrook et al., 1989). Restriction and DNA modifying enzymes were obtained from Roche Molecular Biochemicals (Almere, The Netherlands). PCR was performed with *Pwo* DNA polymerase as described by the supplier using a Perkin-Elmer GeneAmp PCR 2400 thermocycler. Oligonucleotides were synthesized by Baseclear (Leiden, The Netherlands). Genetic manipulations of *H. polymorpha* were performed as detailed before (Faber et al., 1992; Faber et al., 1994b). Double stranded DNA sequencing was carried out using both a LiCor automated DNA sequencer (LiCor inc., Lincoln, NE) with dye-primer chemistry and an ABI 310 Genetic Analyser (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), using dye-terminator chemistry.

Alignments of amino acid sequences were produced using Clustal_X (Thompson et al., 1997). The TBLASTN algorithm (Altschul et al., 1990) was used to search the DDBJ/EMBL/GenBank database (release of February, 2001) for protein sequences showing similarity to the putative *HpPUR7* and *HpPUR8* gene products. The sequence data for *HpPUR7* (909 bp) are available from EMBL/GenBank/DDBJ under accession number AY034035, those for *HpPUR8* (1,485 bp) under accession number AY033990.

Chromosomal DNA was extracted from cells grown overnight on YPD, as described by Sherman et al. (1986), but included a protein precipitation step using 1.5 M of NaCl prior to DNA precipitation. Southern blot analysis was performed using the ECL direct nucleic acid labeling and detection system, as described by the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, England).

Construction of plasmids

To facilitate cloning, an *Asp*718 site was introduced upstream of the *HpPUR7* gene and its promoter by PCR, using primer 5'-CAA**GGTACC**AACACAGATCGCC-3' and the M13/pUC reverse sequencing primer, with as template pHS5-*ADE11*, that fully complements the *ade11* mutant. The resulting 2.1 kb PCR product was cloned as an *Asp*718-*Bam*HI fragment into pBluescript SK^+ (Stratagene Inc., San Diego, CA), digested with *Asp*718 and *Bam*HI, resulting in plasmid pHA1. Subsequently, a 46 bp

fragment containing the *Bam*HI to *Sal*I fragment of the multiple cloning site of pBluescript SK$^+$ was inserted into the 6.7 kb *Bam*HI-*Sal*I fragment of plasmid pHIPX4-PAS3 (Kiel et al., 1995), resulting in pHIPX4-C. Finally, the 1.9 kb *Not*I-*Bgl*II fragment of pHIPX4-C, containing the alcohol oxidase promoter (P_{AOX}), a short multiple cloning site, and the terminator region of the amine oxidase (*AMO*) gene (T_{AMO}), was cloned into pHA1 digested with *Bam*HI and *Not*I. This resulted in plasmid pHIPA4 (see Fig 1).

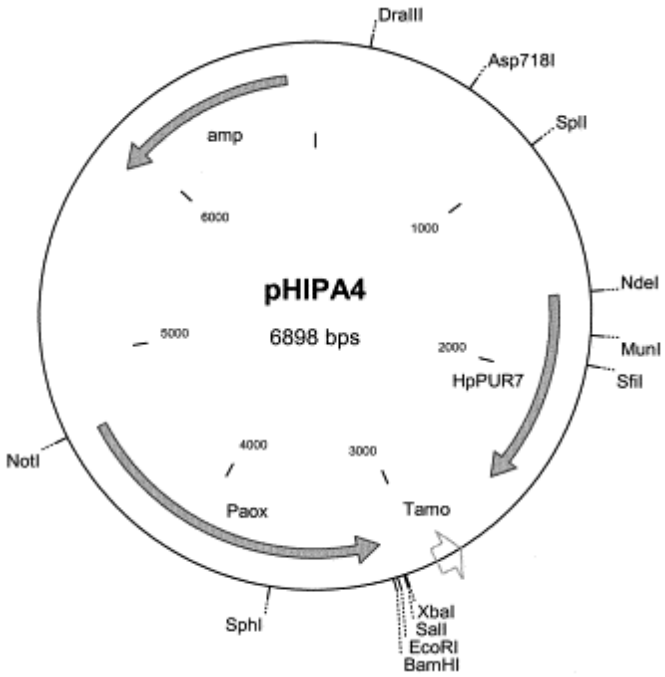

Fig 1*. Physical map of integrative plasmid pHIPA4. Indicated are:* amp*, ampicillin resistance gene;* P_{aox}*,* H. polymorpha *alcohol oxidase promoter;* HpPUR7*,* H. polymorpha PUR7 *gene;* T_{amo}*,* H. polymorpha *amine oxidase terminator.*

A plasmid for overexpression of the *H. polymorpha AMO* gene was constructed as follows: The *AMO* gene was cloned as a *Nhe*I (Klenow-treated) – *Sph*I fragment of pGF71 (Faber et al., 1994a) into pHIPA4 digested with *Bam*HI (Klenow-treated) and *Sph*I, thereby placing *AMO* under control of $P_{AOX.}$ This resulted in plasmid pHIPA4-AMO. An overexpression vector for the *H. polymorpha* catalase gene (*CAT*) was constructed by cloning a 1.5 kb PCR product obtained using primers 5'-AGA AAG CTT ATG TCA AAC CCC CCT G-3' and 5'-TCT **GTC GAC** GAT TAT ATT TTG GAT GGA G-3' as a blunt-*Sal*I fragment into *Bam*HI (Klenow fill in)-*Sal*I digested pHIPA4. The resulting plasmid, containing the *CAT* gene placed under the transcriptional control of P_{AOX} , was designated pHIPA4-CAT.

To identify the DNA fragment complementing mutant KL110 (van Dijk et al., 2002), a 2.2 kb *Xba*I fragment of the original 6.3 kb complementing fragment was cloned into

the *Xba*I site of pHS5. This resulted in pHS5-PUR8, that failed to complement KL110, but was demonstrated to contain the putative *HpPUR8* gene by sequencing.

Biochemical procedures

Crude extracts were prepared as described (Baerends et al., 2000). Extracts prepared from an equivalent of 0.3 OD units of cells were subjected to SDS-PAGE and Western blotting. Western blots were decorated using polyclonal rabbit anti-AMO or anti-CAT antiserum. AMO and CAT activities were assayed as described (Zwart et al., 1980; Lück, 1963).

Electron microscopy

Whole cells were prepared for electron microscopy and immunocytochemistry as described (Waterham et al., 1994). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against AMO and CAT and gold-conjugated goat anti-rabbit antibodies (GAR-gold) according to the instructions of the manufacturer (Amersham Corp., Arlington Heights, IL, USA).

Results

Cloning and sequence analysis of the putative *H. polymorpha PUR7* and *PUR8* genes

In order to enhance the possibilities for genetic manipulation of *H. polymorpha*, we set out to isolate genes involved in purine biosynthesis to create *ade*$^-$ auxotrophs. By coincidence, we discovered a putative *HpPUR8* gene that appeared to be present on the chromosomal insert of a clone from our genomic DNA library of *H. polymorpha* (Tan et al., 1995). This particular plasmid functionally complemented the *ass3* mutant that was defective in the assembly of peroxisomal alcohol oxidase (van Dijk et al., 2002). The putative *H. polymorpha PUR8* gene encoded a protein of 495 amino acids, with a calculated Mw of 55,931 Da. Its protein product, HpPur8p, showed high sequence similarity to Pur8 proteins of several eukaryotic organisms, including *S. cerevisiae* (Ade13p, 80% identity, 88% similarity), *Homo sapiens* (63% identity, 76% similarity) and *Caenorhabditis elegans* (42% identity, 60% similarity). A sequence alignment reveals a high degree of conservation throughout the entire sequence (Fig 2). As expected, *H. polymorpha* Pur8p displays a fumarate lyase signature at position 298-307 (GSSAMAYKRN). Based on these similarities, we

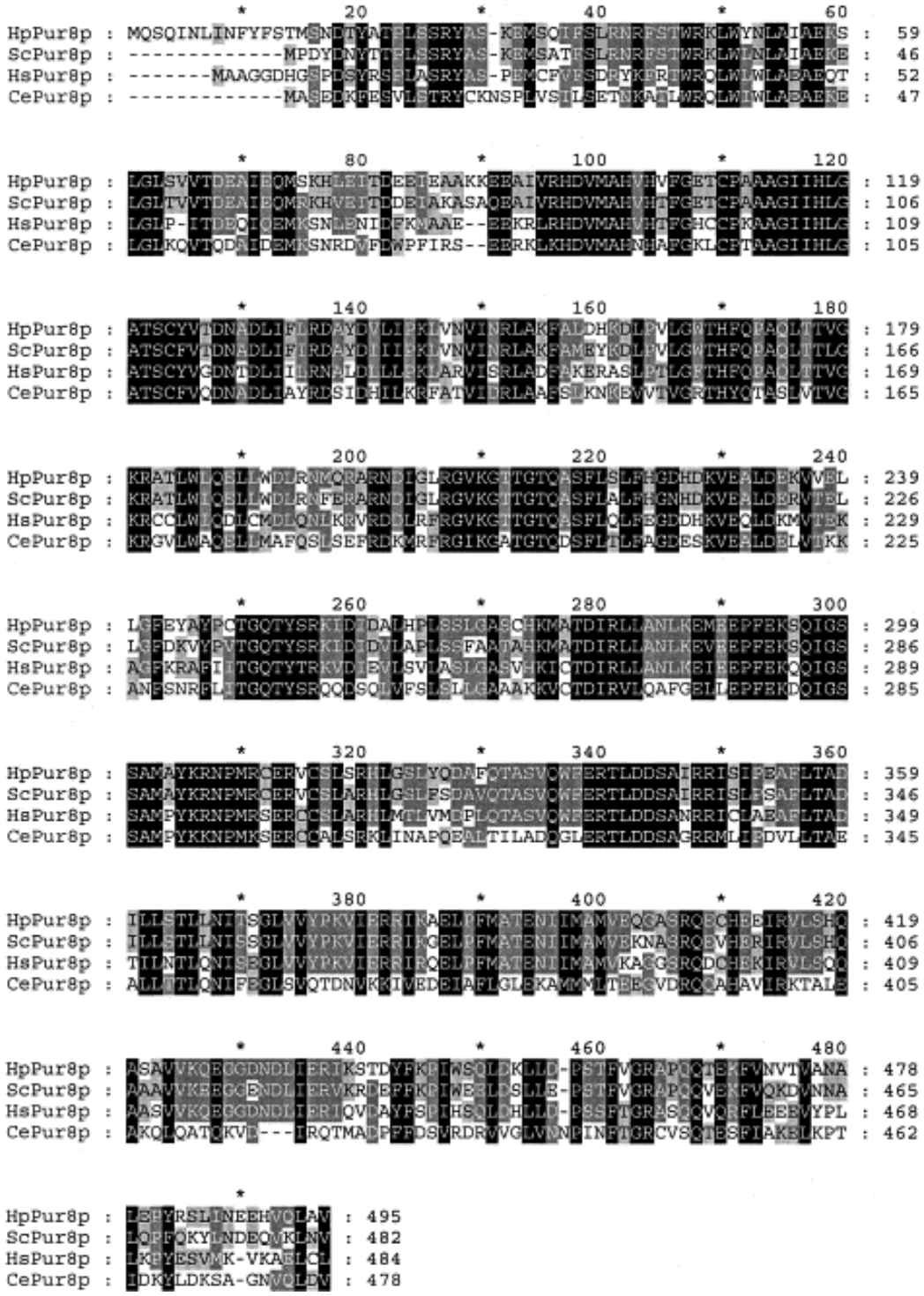

***Fig 2**. Alignment of the amino acid sequences of putative Pur8 proteins of* H. polymorpha *(Hp),* Saccharomyces cerevisiae *(Sc),* Homo sapiens *(Hs), and* Caenorhabditis elegans *(Ce). The one-letter code is shown. Gaps are introduced to maximize the similarity. The shading of amino acids indicates degree of conservation: black box: conserved in four out of four sequences; dark grey: conserved in three out of four sequences; light grey: conserved in two out of four sequences. This figure was prepared using the Genedoc program (Nicholas and Nicholas, 1997, http://www.psc.edu/biomed/genedoc/).*

conclude that this protein probably catalyses the eighth step in purine biosynthesis in *H. polymorpha*.

A screen of the available *H. polymorpha ade* mutants (Gleeson and Sudbery, 1988) did not result in identification of a mutant phenotype that was functionally complemented by *HpPUR8*. Therefore, we used the reciprocal approach to isolate the gene that functionally complemented selected *ade* mutants, using our genomic DNA library. Strains from two out of the twelve available complementation groups (Gleeson and Sudbery, 1988), namely *ade11* and *ade12*, displayed the typical red color also observed for *S. cerevisiae ade1* and *ade2* mutants upon growth of cells in adenine-limiting conditions. Of these, *H. polymorpha ade11.1*, that is presumed to accumulate 5-aminoimidazole-4-carboxylic acid ribonucleotide, was selected to isolate the corresponding gene by functional complementation. The original *ade11.1* mutant was backcrossed three times with strain *H. polymorpha* NCYC495 *leu1.1* prior to transformation with a *H. polymorpha* genomic library in pYT3, in order to eliminate possible secondary mutations accumulated during mutagenesis. After transformation and plating on YND containing adenine, leucine prototrophic colonies were replica-plated on medium lacking adenine to select for adenine prototrophs. A plasmid (designated pYT3-ADE11) containing a 10 kb genomic DNA fragment was rescued from a complemented mutant strain. Initial DNA sequence analysis of the complementing part of the insert revealed the presence of an ORF, encoding a protein of 303 amino acids, with a calculated Mw of 33,890 Da. A database search revealed high sequence similarity to several Pur7 proteins of other yeast species, including *Pichia jadinii* (70% identity, 82% similarity), *S. cerevisiae* (Ade1p, 67% identity, 80% similarity) and *Candida maltosa* (63% identity, 78% similarity). Sequence alignment of these proteins reveals a high degree of conservation throughout the entire sequence (Fig 3). The *H. polymorpha* protein contains two SAICAR synthetase 1 signatures at positions 113-127 (LVPLEAIVRGYITGS) and 212-220 (LADTKFEFG).

Construction of strains overproducing AMO or CAT

The original *H. polymorpha ade11.1* grew very poorly on methanol (final OD_{660} 1.0 versus 3.5 for WT after overnight culturing), a phenomenon that appeared to be due to a partial peroxisomal import defect of catalase protein (data not shown). Therefore, this mutant was backcrossed three times with NCYC495 *leu1.1* to obtain strain *H. polymorpha* NCYC495 *ade11.1 leu1.1.* This strain grew on methanol at WT rates and catalase was normally localized in peroxisomes of these cells. This indicates that the observed effects on growth and catalase localization are not linked to the *ade11.1* phenotype, but instead are second-site mutations. To allow full

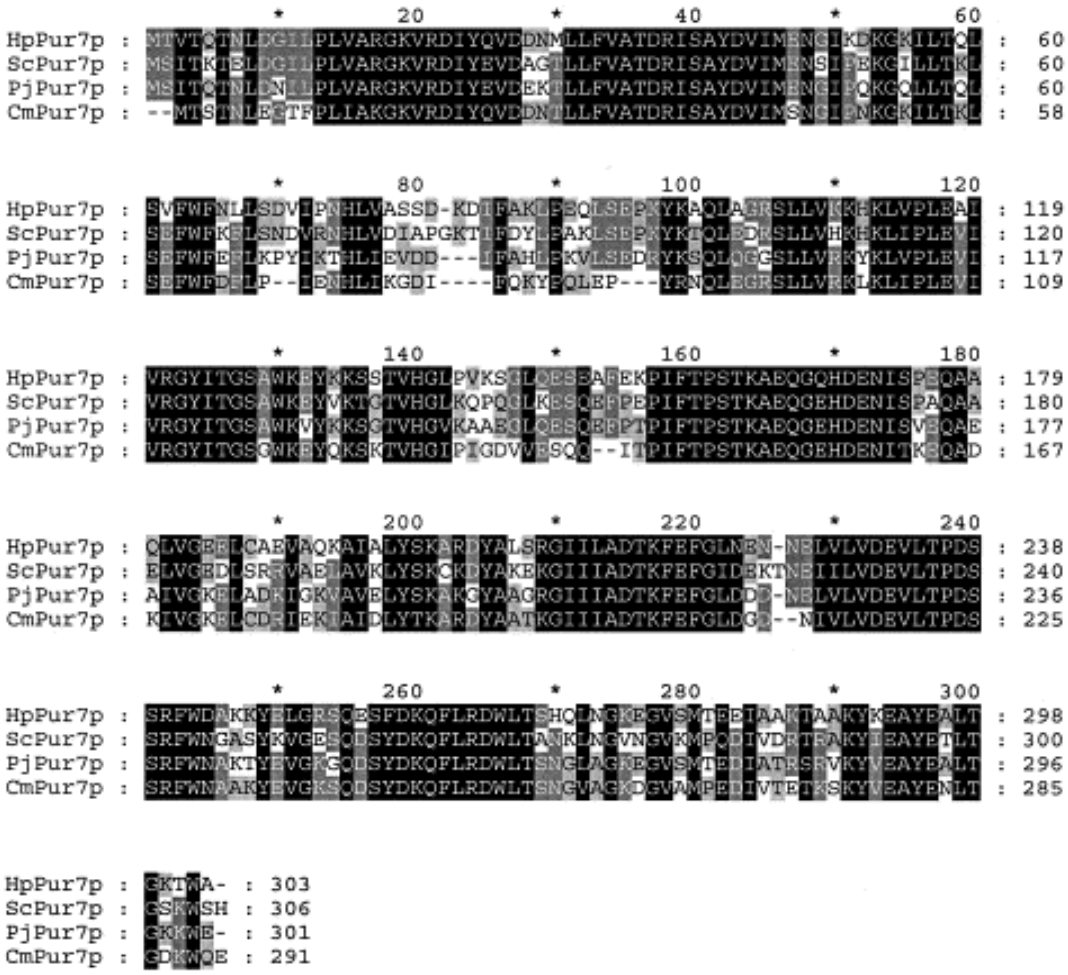

***Fig 3**. Alignment of the amino acid sequences of Pur7 proteins of* H. polymorpha *(Hp),* Saccharomyces cerevisiae *(Sc),* Pichia jadinii *(Pj), and* Candida maltosa *(Cm). For details see legend to Fig 2. In higher eukaryotes, including man, the corresponding* PUR7 *gene encodes a multifunctional protein, the N-terminal half of which is homologous to SAICAR synthetases, the C-terminal half being the catalytic subunit of phosphoribosylaminoimidazolecarboxylase (AIR carboxylase, EC 4.1.1.21). Therefore, only the functional homologues of several yeast species are depicted in this figure.*

exploitation of the new auxotrophic marker, we crossed this strain with NCYC495 *ura3* to yield strain NCYC495 *ade11.1 leu1.1 ura3*. This strain is suitable for independent introduction of three expression cassettes, each using one of the auxotrophic markers for selection. We constructed vector pHIPA4 to mediate overexpression of genes under control of P_{AOX} while using the *ADE11* gene as selection marker in an *ade11.1* background. The application of pHIPA4 to mediate protein overproduction was analyzed using *H. polymorpha* amine oxidase (AMO) and catalase (CAT) as model proteins.

For integration at the *AOX* locus, plasmids pHIPA4-AMO and pHIPA4-CAT were linearized by digestion with *Sph*I and subsequently used to transform strain *H. polymorpha* NCYC495 *ade11.1 leu1.1 ura3*. The transformation procedure yielded

approximately 1,000 transformants per µg of linearized DNA. After three days of growth on YND plates supplemented with leucine and uracil, white Ade$^+$ colonies appeared on plates containing cells transformed with the linearized plasmids. To confirm proper integration of the constructs at the P_{AOX} locus, Southern blotting was performed using chromosomal DNA isolated from selected Ade$^+$ transformants. Out of 20 pHIPA4-AMO transformants tested, 15 showed insertion of the construct at the P_{AOX} in a single copy (data not shown). Two transformants contained two copies of the construct. Multi-copy integrants were not observed. Three transformants still contained the WT fragment of P_{AOX} suggesting that integration had occurred at a locus other than P_{AOX}. A characteristic Southern blot of two selected Ade$^+$ designated transformants obtained using pHIPA4-AMO is shown in Fig 4. One of these, NCYC495 *ade11.1 leu1.1 ura3*::[P_{AOX}*AMO*]$_1$ contains a single copy of the expression cassette, the second one, NCYC495 *ade11.1 leu1.1 ura3*::[P_{AOX}*AMO*]$_2$ contains two copies. Both strains were used for further analysis. Similar integration frequencies were obtained for pHIPA4-CAT transformed cells (data not shown). This suggested that the construct containing the full-length promoter of the *ADE11* gene

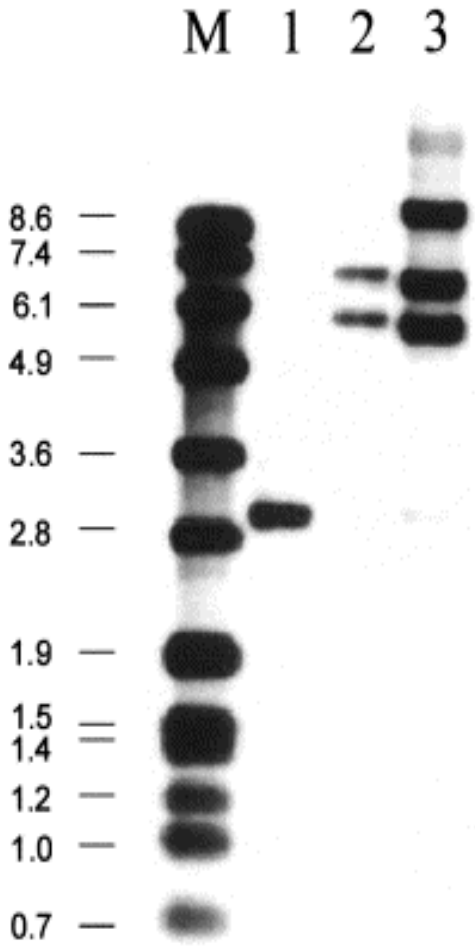

Fig 4. *Southern blot analysis of selected* H. polymorpha *pHIPA4-AMO transformants. Chromosomal DNA was digested using* Eco*RI, electrophoresed and blotted onto Hybond-N$^+$ nylon transfer membrane (Amersham Pharmacia Biotech) by capillary blotting. Bacteriophage SPP1 DNA digested with* Eco*RI was used as molecular weight marker (fragment sizes indicated in kb). The blots were hybridized using the 1.5 kb* Hind*III*/Not*I fragment of pHIPX4 (Gietl et al., 1994) (containing the P_{AOX} locus) mixed with a small amount of SPP1 DNA digested with EcoRI as a probe. M: Molecular weight marker, Lane 1: H. polymorpha NCYC495 ade11.1 leu1.1 ura3, Lane 2: NCYC495 ade11.1 leu1.1 ura3::[P_{AOX}AMO]$_1$, Lane 3: NCYC495 ade11.1 leu1.1 ura3::[P_{AOX}AMO]$_2$.*

complemented the mutant efficiently. Double or multiple-copy integration events, that can be specifically selected for when a construct is used that complements the mutant phenotype poorly when present in a single copy (Agaphonov et al., 1999), did not occur at high frequencies. Our results indicate that plasmid pHIPA4 is pre-eminently suited for the construction of mutants containing a single copy of the overexpression cassette.

The production levels of AMO and CAT protein in the overproducing strains are shown in Fig 5. The data indicate that in the host strain AMO protein is solely synthesized in cells grown in the presence of the AMO-inducing substrate, ethylamine, but not in the presence of ammonium sulphate, independent of the carbon source used for growth (Fig 5A). In the single-copy transformant AMO protein

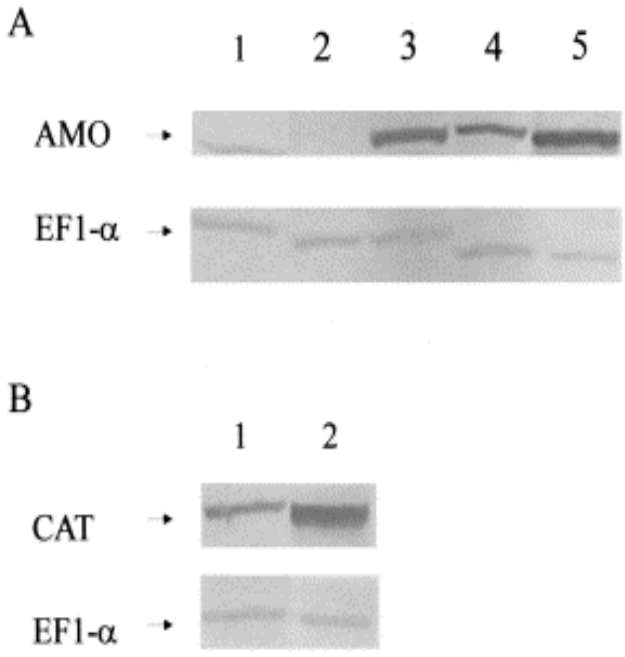

Fig 5*. Western blot analysis of strains overproducing AMO (A) or CAT (B). Per lane an amount of protein corresponding to 0.3 OD units of cells was loaded; as loading control the level of the constitutive elongation factor 1-α (EF1α) was used. Blots were decorated with polyclonal α-AMO, α-CAT, or α-EF1α antibodies. Detection was performed using secondary alkaline phosphatase conjugated goat-α-rabbit antibodies and NBT-BCIP substrate.*
A: Lane 1: WT H. polymorpha *NCYC495* ade11.1 leu1.1 ura3 *cells grown on glucose/ammonium sulphate; Lane 2: WT cells grown on methanol/ammonium sulphate; Lane 3: WT cells grown on glucose/ethylamine; Lane 4: NCYC495* ade11.1 leu1.1 ura3*::[P_{AOX}AMO]$_1$ cells grown on methanol/ammonium sulphate; Lane 5: NCYC495* ade11.1 leu1.1 ura3*::[P_{AOX}AMO]$_2$ cells grown on methanol/ammonium sulphate.*
B: Lane 1:WT H. polymorpha *NCYC495* ade11.1 leu1.1 ura3 *cells grown on methanol/ammonium sulphate; Lane 2: NCYC495* ade11.1 leu1.1 ura3*::[P_{AOX}CAT]$_1$ cells grown on methanol/ammonium sulphate.*

is detected upon growth of cells on methanol/ammonium sulphate at levels comparable to those observed in the amine-induced host strain. As expected, enhanced levels are observed in extracts prepared from the two-copy transformant (Fig 5A). Comparable results were obtained for CAT protein, that is present in

enhanced levels in extracts prepared from the single copy transformant compared to the control host strain (Fig 5B).

Immunocytochemistry revealed that the rate of AMO and CAT overproduction exceeded the maximum uptake capacity for these proteins of the peroxisomes present in the cells, in that the proteins were found in both the organelles and the cytosol (shown for CAT, Fig 6A). Remarkably, CAT protein was also observed in mitochondria (Fig 6B), a phenotype that was observed before in some of the *ass* mutants (van Dijk et al., 2002).

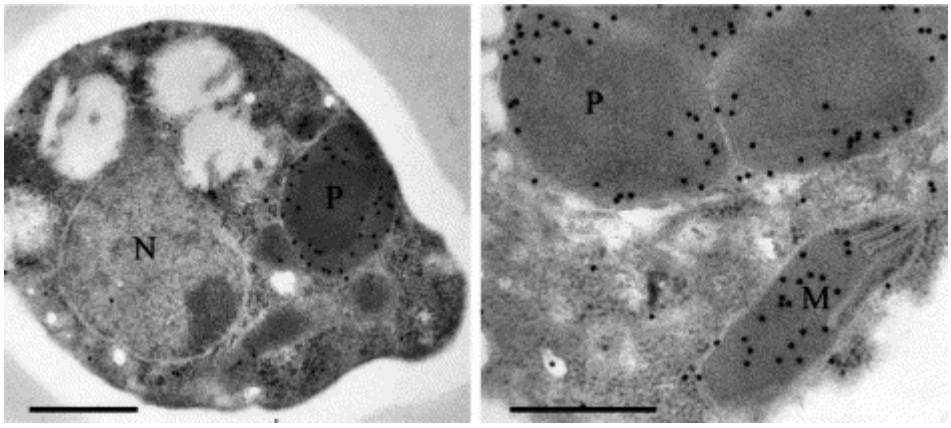

Fig 6*. Immunocytochemical experiments to demonstrate the location of CAT protein, produced in methanol-grown NCYC495* ade11.1 leu1.1 ura3*::[P_{AOX}CAT]$_1$ cells. α-CAT dependent labeling is observed on peroxisomes and cytosol (Fig 6, left) but also on mitochondria (Fig 6, right).*
P*, peroxisome,* N*, nucleus,* M*, mitochondrion,* V*, vacuole. Bar represents 0.5 µm.*

Discussion

In this paper we report the isolation of the putative *H. polymorpha PUR7* and *PUR8* genes that are involved in purine biosynthesis in this organism. An expression vector containing the putative *HpPUR7* gene, pHIPA4, was designed and successfully used for production of the homologous peroxisomal matrix proteins amine oxidase (AMO) and catalase (CAT). The *H. polymorpha PUR7* gene was cloned by functional complementation of the *ade11* mutant strain. The red colonies that the *ade11* mutant forms when grown on rich solid media under adenine-limiting conditions, enable an easy distinction between complemented and non-complemented *ade11* colonies (see below). Previously, only three other selectable genetic markers were available in *H. polymorpha* (*leu1.1*, *trp3*, and *ura3*). Therefore, the additional possibilities provided by the *ade11* mutant will be of value for future applied and fundamental

studies using this organism. For this reason we constructed the threefold auxotrophic mutant, NCYC495 *ade11.1 leu1.1 ura3*, and the expression vector pHIPA4. The feasibility of this system is convincingly demonstrated by the successful overproduction of the homologous proteins AMO and CAT.

Several authors have reported on plasmid-based systems for overproduction of proteins in methylotrophic yeast species. These systems have been used successfully for production of both homologous and heterologous polypeptides. Furthermore, very strong and well-regulated promoters have been isolated (e.g. promoters of the alcohol oxidase, dihydroxyacetone synthase, and formate dehydrogenase genes, for review see van Dijk et al., 2000). The use of these systems has led to the successful overproduction of various proteins, e.g. *Aspergillus niger* glucose oxidase, *S. cerevisiae* invertase, *Aspergillus aculeatus* cellulase-I, *Homo sapiens* urokinase, and hepatitis B antiserum (reviewed in (van Dijk et al., 2000)).

The *H. polymorpha ade11.1 leu1.1 ura3* mutant, together with the newly developed expression vector pHIPA4, provides an additional tool to enhance the possibilities to introduce foreign or homologous genes into this organism. All three selectable markers have now been used successfully for genetic modification of *H. polymorpha*. In addition, the zeocin resistance marker has recently been used in *H. polymorpha* to construct an *AOX* disruption strain (Waterham et al., 1997). Use of this dominant marker creates the possibility of introducing a gene into a prototrophic strain. Thus, the tools are now available for independently introducing four expression cassettes into *H. polymorpha*, either by genomic integration or as replicating plasmids. Genomic integration generally generates stable mutants, as indicated by the fact that we routinely grow transformants on rich media. During these procedures we never observe any significant phenotypic reversion.

Analysis of transformants obtained using the expression vector pHIPA4 revealed that the integration cassette typically inserts in a single copy into the genome of the *ade11* mutant. This indicates that the adenine auxotrophic phenotype is efficiently complemented upon insertion of a single copy of the homologous *HpPUR7* (*ADE11*) gene. The expression system described here is therefore particularly suited for obtaining modest production levels of selected proteins. This was shown for the *H. polymorpha* AMO and CAT proteins. Especially in those cases where high-level overproduction of proteins might cause artifacts, the system for obtaining moderate expression levels described here posseses distinct advantages. However, it remains possible to utilize the *HpPUR7* gene for multicopy integration e.g. by reducing the

complementation efficiency of the mutant phenotype (Agaphonov et al., 1999). This can be achieved by using plasmids with poor complementing activity, e.g. plasmids that contain fragments of the promoter of the complementing gene, or by using heterologous instead of homologous markers.

Using electron microscopy we examined the subcellular fate of the overproduced proteins in *H. polymorpha*. Two separate pathways for the import of peroxisomal matrix proteins have been identified. The PTS1 pathway is used for the import of most of the matrix proteins, e.g. AO and CAT (de Hoop and AB, 1992; Subramani, 1993). The alternative PTS2 pathway is used for import of only a small number of peroxisomal matrix proteins, e.g. AMO. Efficient targeting of AMO to the peroxisome requires induction of the PTS2 pathway (Faber et al., 1995). In the AMO overproducing cells analyzed here, AMO is present in both peroxisomes and cytosol. As shown before, in *H. polymorpha* the PTS2 import machinery is severely repressed in the presence of ammonium and has to be induced by amines (Faber et al., 1994a). Therefore, the observed dual location of AMO protein upon overproduction is in agreement with earlier data (Faber et al., 1994a; Faber et al., 1995). In the CAT overproducing cells, a similar localization of the overproduced protein was observed. Although a large portion of CAT is found in the peroxisomal matrix, a significant amount is mislocalised to the cytosol and mitochondria. This result shows that even though the PTS1 import route, which is used for CAT import into peroxisomes, is fully active under the conditions used, a large import defect is observed. Mislocalisation of CAT to mitochondria was also observed in some of the AO assembly (ass) mutants isolated in our lab (van Dijk et al., 2002). At this point, we can only speculate on the mechanisms underlying this phenomenon.

Recently, approximately 50% of the genome of *H. polymorpha* has been sequenced in an effort of Genoscope (National Centre for Sequencing, France; www.genoscope.cns.fr) (Blandin et al., 2000). During an initial search we identified several partial open reading frames sharing homology with *PUR* genes from *S. cerevisiae*. In the course of this search we found the ORF of *ADE13* (*HpPUR8*). Furthermore, the coding sequences for (part of) the putative *ADE2*, *ADE3*, *ADE5/7*, *ADE6*, *ADE8*, *ADE12*, *ADE16*, and *ADE17* homologues of *H. polymorpha* were found, using the *S. cerevisiae* proteins as queries. We plan to combine this sequence information with the results presented above to set up a synthetic lethality screening system (Guarente, 1993), that can be used to study the interaction of gene products, e.g. using plasmids containing genes involved in purine biosynthesis.

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Chapter 3

Hansenula polymorpha Pex3p is a peripheral component of the peroxisomal membrane

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Abstract

Hansenula polymorpha Pex3p plays an essential role in the biogenesis and maintenance of the peroxisomal membrane. In the initial report, bakers yeast Pex3p was suggested to represent an integral component of the peroxisomal membrane, containing one membrane spanning region that exposes the N-terminus of the protein into the organellar matrix. Biochemically, HpPex3p behaved like an integral membrane protein, as it was resistant towards high salt and carbonate treatment. However, urea fully removed Pex3p from the membrane under conditions that the integral membrane protein Pex10p was resistant to this treatment. Additional experiments including protease protection assays and pre-embedding labeling experiments on purified organellar fractions from cells that produced Pex3p's carrying myc epitopes at various selected locations in the protein, revealed that invariably all myc tags were accessible for externally added proteases and antibodies, independent of the presence of detergents. Also, overproduction of Pex3p failed to demonstrate the typical integral membrane protein structures in fracture faces of freeze fractured peroxisomes. Taken together, our data suggest that HpPex3p does not span the peroxisomal membrane, but instead is tightly associated to the cytosolic face of the organelle where it may be present in focal protein clusters.

Introduction

Peroxisomes are ubiquitous subcellular compartments characterized by the presence of enzymes, which produce and degrade H_2O_2. Peroxisomes are recognized as a class of versatile organelles, which play an essential role in the cellular metabolism in all eukaryotes, including man (for reviews, see Gould and Valle, 2000; Titorenko and Rachubinski, 2001).

In yeasts, peroxisomes are predominantly involved in the primary metabolism of specific carbon and/or nitrogen sources used for growth (van der Klei and Veenhuis, 1997). In the past few years we have isolated various peroxisome-deficient (*pex*) mutants of the methylotrophic yeast *Hansenula polymorpha* and cloned sixteen of the corresponding genes (Veenhuis et al., 2001). Previously, we reported the cloning and partial characterization of one of these genes, *PEX3*, which encodes a 52 kDa protein essential for peroxisome biogenesis, and showed that Pex3p plays a key role in the biogenesis and maintenance of the peroxisomal membrane (Baerends et al.,

1996). The crucial importance of Pex3p in peroxisome biogenesis is underlined by the absence of peroxisomal membrane remnants (`ghosts') in a *pex3* deletion strain. A comparison of the putative topologies reported for Pex3p homologues from several organisms shows remarkable differences. For most of the known Pex3p homologues the N-terminus of the protein was shown to reside in the peroxisomal matrix, while the C-terminus protrudes into the cytosol. On the other hand, Ghaedi *et al*. recently reported on the topology of *Rattus norvegicus* Pex3p, which exposes both N- and C-termini into the cytosol (Ghaedi et al., 2000).

In HpPex3p, a short stretch of positively charged amino acid residues located at the N-terminus of the protein (Arg^{11} X Lys Lys Lys^{15}), may play a role in sorting of the protein (Baerends et al., 2000). Comparable signals were observed in Pex3p's of other organisms as well as in ScPex15p (Elgersma et al., 1997; Wiemer et al., 1996). Moreover, HpPex3p also contains a sequence that resembles the mPTS (Peroxisomal Targeting Signal for membrane proteins) presented for PMP47 of *Candida boidinii* (Dyer et al., 1996). The function of this sequence is not yet clear; however, proper sorting requires additional information than solely the positively charged amino acids 11-15. This was clear from experiments which demonstrated that the first 37 N-terminal amino acids were capable to efficiently target a reporter protein to the peroxisomal membrane (Baerends et al., 1996), whereas catalase, fused to the N-terminal 16 amino acids of Pex3p, was mislocalized to the ER and the nuclear membrane but did not reach its target membrane. These data lend support to the view that the ER may play a role in targeting of HpPex3p.

In the course of our studies to further delineate the precise function of HpPex3p in peroxisome biogenesis, we obtained evidence that the topology of the protein may deviate from that proposed for its orthologue in *S. cerevisiae*. Further understanding of the role of HpPex3p is critically dependent on the detailed understanding of the insertion of the protein in the membrane. This prompted us to study the topology of HpPex3p in more detail, using various methods. The combined results, which suggested that HpPex3p is tightly associated to the cytosolic face of the peroxisomal membrane, are presented in this chapter.

Experimental procedures

Micro-organisms and growth conditions

A list of *Hansenula polymorpha* strains used in this study is presented in Table I. All *H. polymorpha* strains were grown in batch cultures at 37°C on mineral medium (van Dijken et al., 1976), supplemented with 0.5% glucose or 0.5% methanol as carbon source, in the presence of 0.25% ammonium sulfate as nitrogen source. When plasmid-containing cells were cultured, reduced amounts of yeast extract (up to 0.02%) were added, to prevent loss of plasmids. For growth on agar plates, all media were supplemented with 1.5-%-granulated agar. *Escherichia coli* DH5α (*supE*44 *ΔlacU*169 (ϕ80*lacZΔ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) (Sambrook et al., 1989) was used for recombinant DNA procedures and was grown on LB-medium supplemented with the appropriate antibiotics.

DNA procedures

H. polymorpha was transformed by electroporation (Faber et al., 1994). Recombinant DNA manipulations were performed essentially as described (Sambrook et al., 1989). Site-directed mutagenesis of *PEX3* and *PEX8* was performed using the polymerase chain reaction and *Pwo* polymerase according to the instructions of the supplier (Roche Molecular Biochemicals, Almere, The Netherlands). The oligonucleotides used in this study are listed in Table II and were obtained from Eurogentec (Seraing, Belgium). Biochemicals were obtained from Roche Molecular Biochemicals.

Construction of myc-tagged variants of HpPex3p and HpPex8p

In order to tag Pex3p with the myc epitope (EQKLISEEDL, (Evan et al., 1985)), we amplified mutant *PEX3* alleles by PCR using oligonucleotides, which introduced an endonuclease restriction site and the sequence encoding the myc epitope into *PEX3*. Chimeric *PEX3*-myc fragments were amplified by using pBS-HP*PEX3* (2.7kb) as template and the primer combinations: 5pex3-myc / pex3-1B, N180pex3 / 3pex3-myc, inpex3-myc / pex3-1B, and 3mycin4 / pex3-1A (see Table II). This amplification introduced the sequence encoding the myc tag at the 5' and 3' ends of *PEX3*, and replacing the amino acid residues at positions 102-111, and 214-223, respectively. The PCR product of 5pex3-myc / pex3-1B was subcloned as a *Bgl*II/*Sal*I fragment into the 2.9 kb *Bgl*II/*Sal*I fragment of pBS-*PEX3* (Kiel et al., 1995), yielding pRBG49. The PCR product of N180pex3 / 3pex3-myc was subcloned as a *Hind*III/*Sal*I

Table I. Strains and plasmids used in this study

Strains/plasmids	Relevant properties	Reference
***E.coli* strains**		
DH5α	*supE*44 *ΔlacU*169 (ϕ80*lacZΔ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(Sambrook et al., 1989)
***H. polymorpha* strains**		
NCYC495 *leu1.1*	Wild type (WT)	(Gleeson and Sudbery, 1988)
RBG1	*Δpex3 leu1.1*	(Baerends et al., 2000)
WT::P_{AOX}*PEX3*^{1x}	WT *H. polymorpha* containing an additional copy of *PEX3* under control of P_{AOX}	(Baerends et al., 1997)
WT::YEpMOX47	WT *H. polymorpha* overexpressing the *C. boidinii PMP47* gene under control of P_{AOX}	(Sulter et al., 1993)
CT103	*H. polymorpha per8-1 leu1* strain containing the *PEX10* (*PER8*) gene under transcriptional control of P_{AOX}	(Tan et al., 1995)
RBG1[P_{PEX3}*·PEX3·N-myc*]	*Δpex3* containing N-terminally Myc-tagged Pex3p (single copy pRBG61)	This study
RBG1[P_{PEX3}*·PEX3·102-111myc*]	*Δpex3* containing Pex3p with Myc tag replacing amino acid residues 102-111 (single copy pRBG63)	This study
RBG1[P_{PEX3}*·PEX3·214-223myc*]	*Δpex3* containing Pex3p with Myc tag replacing amino acid residues 214-223 (single copy pGJH50)	This study
RBG1[P_{PEX3}*·PEX3·C-myc*]	*Δpex3* containing C-terminally Myc-tagged Pex3p (single copy pGJH2)	This study
RBG46	WT *H. polymorpha* containing stable integration of a single copy of *Stu*I-lineairized pHOR46	This study
HF47	WT *H. polymorpha* expressing $PEX3_{[1-50]}$GFP under control of P_{AOX}	(Baerends et al., 2000)
HF250	WT *H. polymorpha* containing 2 copies of pFEM193 integrated into the P_{AOX}-locus	This study
Δpex8	*Δpex8 leu1.1*	This study
Δpex8::P_{AOX}*PEX8·C-myc*	*Δpex8* containing a single copy of pHIPX4-CmycPex8 at the P_{AOX}-locus	This study
plasmids		
pBluescript II SK$^+$		Stratagene Inc., San Diego, CA
pBluescript II KS$^+$		Stratagene Inc., San Diego, CA
pHIPX4-*PAS3*	*H. polymorpha* expression plasmid with *AOX* promoter containing *Saccharomyces cerevisae PEX3* gene	(Kiel et al., 1995)
pHIPX6	*H. polymorpha* expression plasmid with *PEX3* promoter	(Kiel et al., 1995)
pHIPX6-HPPEX3	pHIPX6 containing the *PEX3* gene	(Kiel et al., 1995)
pHIPX6PEX3ΔC444	pHIPX6 containing N-terminal 443 amino acids of *PEX3* (*Bam*HI/*Sal*I)	This study
pRBG1	pBluescript II SK$^+$ containing 0.5 kb *Sac*I-*Bam*HI fragment of P_{PEX3}	This study
pRBG2	pRBG1 containing 2.3 kb *Bam*HI-*Sal*I fragment of the *URA3* gene	This study
pRBG3	pRBG2 containing 0.1 kb PCR product of the 3'-untranslated region of the *PEX3* gene	This study
pRBG46	pHIPX6_PEX3 (R11E/K13E/K15E)	(Baerends et al., 2000)
pRBG49	pBluescript II KS$^+$ containing PEX3·N-Myc	This study
pRBG50	pBluescript II KS$^+$ containing PEX3·102-111myc	This study
pGJH49	pBluescript II KS$^+$ containing PEX3·214-223myc	This study
pRBG51	pBluescript II KS$^+$ containing PEX3·C-Myc	This study
pRBG52	pBluescript II SK$^+$ containing N180-PEX3·C-Myc	This study
pRBG61	pHIPX6_PEX3·N-Myc	This study
pRBG63	pHIPX6_PEX3·102-111myc	This study
pGJH50	pHIPX6_PEX3·214-223myc	This study
pGJH2	pHIPX6_ PEX3·C-Myc	This study
pHOR46	Self-ligated 7.2 kb *Not*I-*Mlu*I (both Klenow treated) fragment of pFEM152	This study
pFEM152	pHIPX4 containing C-terminally tagged version of *PEX3*	(Faber et al., 2001)
pFEM193	expresses a fusion of ΔN_{50}*PEX3* to GFP under control of P_{AOX}	This study
pBS-PER1tot	pBluescript containing 4.5 kb *Nhe*I-*Xba*I fragment of *PEX8*	This study
pBS-URA3	pBluescript containing 2.3 kb *Bam*HI fragment of *URA3*	This study
pHOR36	pBS-URA3 containing 0.9 kb 3' UTR of *PEX8*	This study
pHOR37	pHOR36 containing 1.6 kb 5' UTR of *PEX8*	This study
pHRP2	Plasmid containing complete *PEX8* ORF	(Waterham et al., 1994)
pHIPX4-CmycPex8	pHIPX4 containing C-terminally Myc-tagged *PEX8* under control of P_{AOX}	This study

fragment into *Hind*III/*Sal*I-linearized pBluescript II SK$^+$, resulting in pRBG52. The 0.4kb *Eco*RI/*Sal*I fragment of pRBG52 was subsequently inserted into the 3.9 kb *Eco*RI/*Sal*I fragment of pBS-*PEX3*, resulting in pRBG51. The PCR product of inpex3-myc / pex3-1B was subcloned as a *Sfu*I-*Sal*I fragment in the 3.2 kb *Sfu*I/*Sal*I fragment of pBS-*PEX3*, thereby replacing the 5'-end of the *PEX3* gene (yielding pRBG50). Finally, the 3mycin4 / pex3-1A PCR product was cloned as a *Bam*HI/*Nco*I fragment into the 3.7 kb *Bam*HI/*Nco*I fragment of pRBG46 (see Table I), yielding pGJH49. The resulting plasmids are listed in Table I.

After subcloning, all fragments were checked by sequence analysis. Plasmids pRBG49, pRBG50, and pRBG51 were digested using *Bam*HI-*Sal*I and the 1.5 kb fragments (containing the chimeric *PEX3* genes) were cloned into *Bam*HI-*Sal*I digested pHIPX6 (Kiel et al., 1995); this yielded pRBG61, pGJH2, and pRBG63, respectively. The 1.4 kb *Hind*III/*Sal*I fragment of pGJH49 was placed into the 6.8 kb *Hind*III/*Sal*I fragment of pHIPX6PEX3ΔC444, yielding pGJH50. The resulting plasmids (see Table I), containing the chimeric genes under control of the *PEX3* promoter (P_{PEX3}), were used to transform the *pex3* knockout strain RBG1 (Baerends et al., 2000). The constructed strains were designated as listed in Table I. All myc-tagged Pex3p proteins functionally complemented the methanol-utilization-deficient *pex3* deletion strain, indicating that they were normally functional as WT Pex3p.

An *H. polymorpha* strain, in which the complete open reading frame of the *PEX8* gene was deleted, was constructed as follows: first, a 4.5 kb *Nhe*I (Klenow-treated)-*Xba*I genomic fragment, containing the *PEX8* gene (Waterham et al., 1994), was cloned in pBluescript II KS+ digested with *Sma*I-*Xba*I (resulting in pBS-*PEX8*). Furthermore, a 2.3-kb *Bam*HI (Klenow-treated) genomic fragment containing the *URA3* gene (Merckelbach et al., 1993), was inserted into *Sma*I-digested pBluescript II SK+, resulting in pBS-URA3 (insert in two orientations). Into pBS-URA3-ori1, cut with *Sal*I (partially) and *Pst*I, a 0.9-kb *Sal*I-*Pst*I fragment containing the 3'-untranslated region of the *PEX8* gene was inserted, which was amplified by PCR using the primers RB24 and RB25(see Table II), and plasmid pBS-*PEX8* as template, resulting in pHOR36. Subsequently a 1.6-kb 5'-untranslated region of the *PEX8* gene was amplified by PCR using the primers reverse and RB23, and pBS-*PEX8* as template, cut with *Hind*III (Klenow-treated)/*Bgl*II and inserted into pHOR36 digested with *Not*I (Klenow-treated)/*Bgl*II, resulting in pHOR37. Finally, a 3.8-kb linear DNA fragment, containing the *URA3* gene flanked by *PEX8* upstream and downstream sequences, was isolated from pHOR37 using *Bam*HI and *Sal*I digestion. This DNA fragment was used to transform *H. polymorpha* NCYC495 [*leu1.1 ura3*] cells; transformants were selected

Table II. Oligonucleotides used in this study

Primer	Sequence
M13 -50 reverse	5'-GCTCGTATGTTGTGTGG-3'
pex3utr3	5'-AGAAGATCTTGATGATGATTGGCAGC-3'
pex3-1B	5'-GTCGTCGACGATATCTAATCAGTATACATGC-3'
pex3-1A	5'-AGAGGATCCCGGGTTCGTTCTCTGTGATAC-3'
N180pex3	5'-GGAAAGCTTATGTCTAGGCGCTCATATC-3'
5'pex3-myc	5'-AGAGGATCCATGGAGCAGAAGTTGATTTCTGAGGAAGACTTGTTCCAATATTGTAGAG-3'
inpex3-myc	5'-GCGATTCGAAAGACAAGAGCAGAAGTTGATTTCTGAGGAAGACTTGACTGATCCAGCGTTGTC-3'
3mycin4	5'-AGAGCCCATGGCCAAGTCCTCCTCAGAAATCAACTTCTGCTCTGCTGGGTCCAGATCCAC-3'
3'pex3-myc	5'-TCTGTCGACTTACAAGTCTTCCTCAGAAATCAACTTCTGCTCAGCATCGAAATTAGAG-3'
RB23	5'-GGGAGATCTGGTAGGAACAAGAAAC-3'
RB24	5'-GGGCTGCAGTATTTAATCTACTCAGTTGG-3'
RB25	5'-CCCGTCGACACACAGCTGGCC-3'
reverse	5'-CAGGAAACAGCTATGAC-3'
Npex8	5'-AGAGGATCCATGCAGCCGTGGTACC-3'
Cmycpex8	5'-TCTCTCGAGCTATAATTTTGCCAAGTCTTCCTCAGAAATCAACTTCTGCTCTTTTTCCTGACTCTCG-3'
KN69	5'-CCCGGATCCATGAAAGAACAAATTAAAAGGCG-3'

for uracil prototrophy and inability to grow on methanol (Mut$^-$). One Mut$^-$ strain was selected which harbored the expected chromosomal alterations, as determined by Southern blot analysis (data not shown). Pex8p was extended at its C-terminus with the myc epitope by site-directed mutagenesis using primers Npex8 and Cmycpex8 (see Table II). pHRP2 (Waterham et al., 1994), containing the *PEX8* ORF, was used as template. After subcloning, the PCR fragment was checked by sequence analysis. The amplified 2 kb fragment was cloned as a *Bam*HI/*Xho*I fragment into *BamH*I/*Sal*I-digested pHIPX4-*PAS3* (Kiel et al., 1995), resulting in pHIPX4-CmycPex8. In this construct, the chimeric *PEX8* gene is under control of the alcohol oxidase promoter (P_{AOX}). *Sph*I-linearized pHIPX4-CmycPex8 was used to transform the *PEX8* deletion strain. Leu$^+$/Mut$^+$ transformants were selected and proper insertion of the construct into the P_{AOX}-locus was verified by Southern blot analysis (results not shown). The regained ability of the transformants to grow on methanol-containing media shows that the Pex8p-myc fusion protein is fully functional. A strain harboring a single copy of the *PEX8*-myc fusion (*Δpex8*::P_{AOX}*PEX8· C-myc*) was selected for use in this study.

Construction of *PEX3· GFP* fusion strains

An *H. polymorpha* wild type strain was constructed in which the endogenous PEX3 promoter regulates the synthesis of the reporter protein Pex3p-eGFP. To this end, a 7.2-kb *Not*I-*Mlu*I (both Klenow treated) fragment of pFEM152 (Faber et al., 2001) was self-ligated, resulting in pHOR46. This plasmid contains only the 3'-end of *PEX3* encoding codons 157-457. Finally, to obtain a stable strain expressing *PEX· GFP* behind P_{PEX3}, *H. polymorpha* NCYC495 [*leu1.1*] cells were transformed with *Stu*I-lineairized pHOR46. Transformants were selected for leucine prototrophy. Correct integration into the *PEX3* locus was confirmed by Southern blot analysis (data not

shown). A single copy integrant was selected for further analysis and designated RBG46.

A WT strain expressing a truncated version of Pex3p, lacking the N-terminal 50 amino acids, was constructed by introducing a *Bam*HI site in front of codon 50 of the *PEX3* gene using primer KN69. The resulting PCR product was cloned as a *Bam*HI/*Sal*I fragment, which resulted in plasmid pFEM193, which expresses a fusion of ΔN_{50}*PEX3* to GFP under control of P_{AOX}. Linearized pFEM193 was used to transform *H. polymorpha* NCYC495 [*leu1.1*] cells as described above. A double copy integrant, designated HF250, was selected for further analysis.

Biochemical methods

Crude extracts (Waterham et al., 1993), protoplasts (van der Klei et al., 1998a) and organellar fractions (van der Klei et al., 1998b) were prepared as described. Protease inhibitors were omitted when organellar fractions were used for protease protection assays. Protease protection assays were performed by incubating organellar fractions (100 μg protein) with various concentrations of proteinase K for 30 minutes on ice, in the absence or presence of 0.1 % Triton X-100. The proteolytic activity was stopped by addition of TCA to a final concentration of 10%. The samples were subsequently analyzed by SDS-PAGE and Western blotting. The behavior of HpPex3p upon extraction of organellar fractions by different reagents was analyzed essentially as described (Elgersma et al., 1997). Protein concentrations were determined using the BioRad protein assay kit (BioRad GmbH, Munich, Germany) using bovine serum albumin as standard. Sodium dodecyl sulfate polyacrylamide gelelectrophoresis (Laemmli, 1970), and Western blotting (Kyhse-Andersen, 1984) were carried out as described. Blots were decorated using the chromogenic (NBT-BCIP) or chemiluminiscent (POD) Western Blotting kit (Roche) using specific polyclonal rabbit antibodies against various *H. polymorpha* peroxisomal proteins or monoclonal mouse α-myc antibodies.

Electron and fluorescence microscopy

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described (Waterham et al., 1994). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific polyclonal antibodies against various peroxisomal proteins or monoclonal α-myc antibodies, and gold-conjugated goat-anti-rabbit or goat-anti-mouse antibodies (Waterham et al., 1994).

Pre-embedding labeling experiments were performed on 30,000x g organellar pellets, prepared from homogenized spheroplasts of methanol-grown cells. These pellets, which predominantly contain mitochondria and peroxisomes, were incubated without further treatment with α-myc or α-Pex3p antibodies. Subsequently, the samples were collected by centrifugation, washed, and incubated with secondary gold-conjugated goat-anti- rabbit or goat-anti-mouse antibodies, followed by fixation in glutaraldehyde and OsO_4, and embedding in Epon 812-resin (Kiebler et al., 1993).

In freeze-etch experiments, methanol-grown cells were frozen in liquid propane and freeze fractured in a Balzer's freeze-etch unit, essentially according to previously described methods (Moor, 1964). Also, lysed spheroplasts of wild type cells were fixed in 4.5% formaldehyde in 0.1M cacodylate buffer, pH 7.2, for 60 min, washed in buffer and subsequently processed for freeze etching.

Strains producing GFP-containing fusion proteins were analyzed by fluorescence microscopy as described (Baerends et al., 2000)

Results

Growth and cell morphology of strains producing myc-tagged Pex3p

For detailed analysis of the topology of *H. polymorpha* Pex3p, various myc tags were introduced. We determined the possible position of putative membrane spans in Pex3p by the algorithms based on the methods of Argos *et al*. (1982); transmembrane helix prediction, Eisenberg *et al*. (1984); prediction of membrane associated α-helices, and Klein *et al*. (1985); hydropathic index of protein. These methods indicate the presence of two or three putative membrane spans in HpPex3p, located at amino acids (aa) 16-36, 159-179, and 366-387, respectively. Based on this information we introduced the tags at the extreme N- and C-termini of the protein, as well as in between the putative membrane spans at positions 102-111, and 214-223. Furthermore, the position of these tags does not coincide with the location of the predicted regions of topogenic information of the protein (Baerends et al., 2000; Dyer et al., 1996).

All constructed *H. polymorpha* strains, RBG1[P_{PEX3}· *PEX3*· *C-myc*], RBG1[P_{PEX3}· *PEX3*· *N-myc*], RBG1[P_{PEX3}· *PEX3*· *102-111myc*], and RBG1[P_{PEX3}· *PEX3*· *214-223myc*] grew like WT cells in batch cultures supplemented with 0.5% methanol as sole carbon source (final optical density at 663 nm of all cultures amounted to approximately 3.0) and contained normal peroxisomes that were morphologically indistinguishable from organelles in WT cells (shown in Fig. 1A for

RBG1[P_{PEX3}· *PEX3*· *N-myc*]). Immunocytochemically, the key enzymes of methanol metabolism, alcohol oxidase, catalase and dihydroxyacetone synthase, were confined to these organelles (data not shown). These results indicated that all four myc-tagged variants of HpPex3p were able to functionally complement the *pex3* deletion strain and hence, that the myc tag did not affect normal Pex3p functioning.

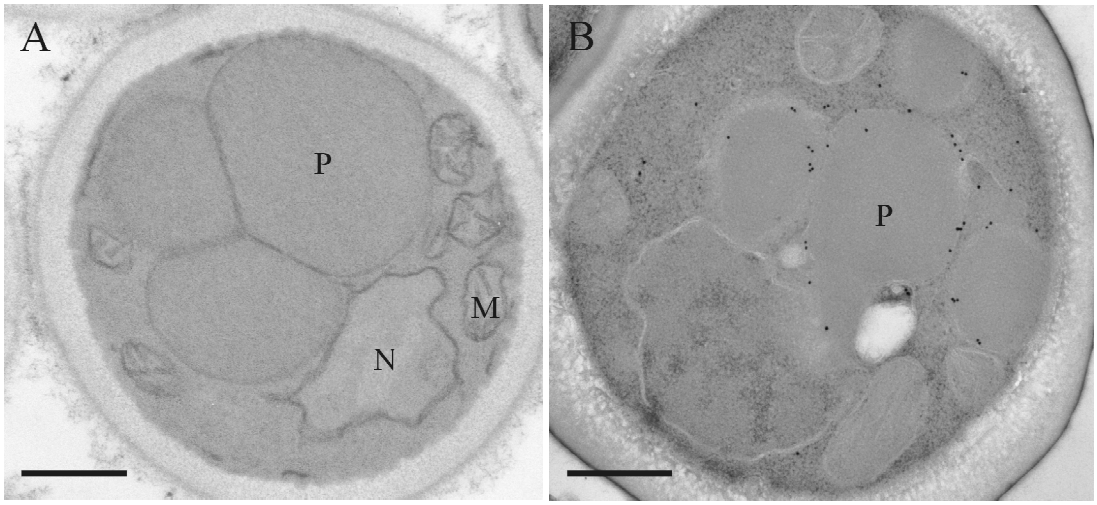

Fig. 1*. Morphology and immunocytochemical analysis of strains producing myc-tagged variants of HpPex3p. A. Overall morphology of RBG1[*P_{PEX3}*.*PEX3. N-myc*] cells, grown for 12 h in batch cultures containing 0.5 % methanol. The morphology equals that of WT* Hansenula polymorpha*, with several peroxisomes present. B. Immunocytochemical analysis of RBG1[*P_{PEX3}*.*PEX3.C-myc*] using α-myc antibodies, showing specific labeling of the peroxisomal membrane (**N**: nucleus, **P**: peroxisome, **M**: mitochondrion). Cells in A were fixed by* $KMnO_4$*, those in B by glutaraldehyde. Bar indicates 0.5 μm.*

The level of Pex3p produced in the various strains was comparable to WT levels, as determined by Western blotting of cell free extracts prepared from cells, grown in batch cultures supplemented with 0.5 % methanol (data not shown). Immunocytochemically, using ultrathin sections of Unicryl-embedded cells of all four strains and monoclonal myc antibodies, the α-myc dependent labeling was confined to the peroxisomal membrane (shown for RBG1[P_{PEX3}.*PEX3*· *C-myc*] in Fig. 1B). These results suggest that in all four strains Pex3p is localized at the peroxisomal membrane, as in WT cells (Baerends et al., 1996).

Biochemical experiments

Protease protection experiments were performed on 30,000x g organellar pellets, prepared from methanol-grown cells of *PEX3* deletion (*pex3)* strains, expressing either one of the four myc-tagged versions of Pex3p. In all four cases, externally added

protease completely degraded the myc-tags, even in the absence of detergent, without leaving any detectable degradation products (Fig. 2). Similar results were invariably obtained using organellar fractions prepared from WT control cells, and α-Pex3p antibodies (see Fig. 2). The matrix enzyme catalase and Pex14p, a membrane-bound

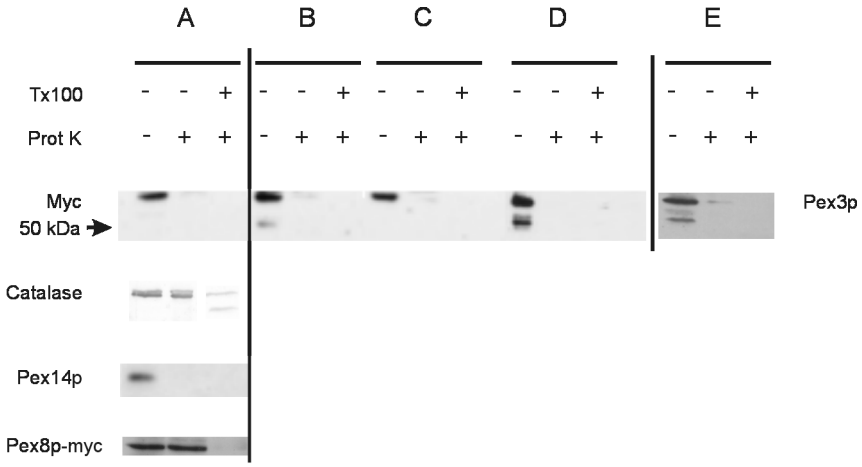

Fig. 2. *Protease protection assay using a 30,000xg organellar pellet prepared from cells of various strains grown for 12 h in mineral media containing 0.5% methanol. 40 μg of protein is loaded per lane. Proteinase K was added to a final concentration of 0.4 mg/ml, Triton X-100 was added at 0.1% (W/V).* ***A****: RBG1[*P_{PEX3}*.PEX3.N-myc], **B**: RBG1[*P_{PEX3}*.PEX3.C-myc], **C**: RBG1[*$P_{PEX3.}$*PEX3.102-111myc]., **D**: RBG1[*$P_{PEX3.}$*PEX3.214-223myc]. Upon addition of proteinase K, myc-tagged Pex3p is completely degraded for all four strains, independent from detergent (**A**-**D**, detection with α-myc antibodies). For the C-terminally tagged proteins, a specific degradation product of approximately 50 kDa is observed in the absence of proteinase K due to endogenous proteolysis(arrow). WT Pex3p behaves similarly (**E,** detection with α-Pex3p antibodies). Pex14p, Pex8p.myc, and catalase were used as controls (only shown for RBG1[*P_{PEX3}*.PEX3. N-myc] cells). Catalase and Pex8p were protected in the absence of detergent but degraded when when detergent was added. Pex14p, a membrane associated protein, was degraded independent from detergent.*

protein located at the cytosolic face of the peroxisomal membrane (Albertini et al., 1997), (Komori et al., 1997), were used as controls. As shown in Fig.2, catalase was protected from degradation in the absence of detergent, whereas Pex14p was completely degraded by the protease, independent of the presence of detergent. This indicated that the peroxisomes present in the 30,000x g pellets were structurally intact. As a further control Pex8p· myc was used. Pex8p is a peroxisomal peroxin associated with the luminal side of the peroxisomal membrane (Waterham et al., 1994). Pex8p· myc fully complemented the *PEX8* deletion strain (not shown) indicating that the fusion protein is normally functional. In organellar pellets, prepared from methanol-grown cells of this strain, Pex8p is resistant to proteolysis by external protease in the absence of detergent indicating that it is protected by the peroxisomal membrane (Fig.

2). Taken together, these data show that all myc tags introduced into HpPex3p are accessible *in vitro* to externally added protease, and therefore are likely to be exposed at the cytosolic face of the peroxisome.

In the protease protection assays a α-Pex3p dependent protein band at approximately 50 KDa was observed, prior to the addition of the protease. An identical band is detected by α-myc antibodies, using organellar fractions containing Pex3p variants that carry the myc epitope in the C-terminal half of the protein, but was never observed using samples of the N-terminally tagged Pex3p versions. Since our polyclonal rabbit α-Pex3p antibodies specifically recognize the C-terminal part of HpPex3p (Baerends et al., 1996), this 50 kDa protein most likely represents a C-terminal fragment of Pex3p (Fig. 2, Lanes B,D,E).

Based on the assumption that low molecular weight degradation products of Pex3p may be formed in case the protein would contain multiple membrane spans, we also analyzed the protease treated samples on SDS-PAGE gradient gels (4-20 % acrylamide). Using such gels, model proteins as small as 6 kDa were readily resolved. However, we never observed any low molecular weight degradation products using these gels, independent of the type of Pex3p used (WT and myc-tagged variants; data not shown). These results suggest that all myc tags were indeed completely degraded by externally added protease.

We also performed extraction experiments to study the nature of Pex3p binding to the peroxisomal membrane. The results, depicted in Fig. 3A, show that WT Pex3p is not extractable from the membrane upon treatment of 30,000x g organellar pellets with 0.1 M TRIS-HCl, pH 8.0, or 1 M NaCl. Also, the protein was almost completely resistant to treatment with 0.1 M Na_2CO_3 (pH 11.5) but became solubilized upon extraction of the samples with 1% CHAPS. In several independent extraction experiments, less than 25% of total Pex3p was found in the supernatant after treatment of the organellar pellets with 0.1 M Na_2CO_3 (pH 11.5). Catalase, used as control, became completely soluble after carbonate treatment and after incubation of the samples in the presence of CHAPS, thus confirming that Pex3p behaves as an integral part of the peroxisomal membrane in the above experiments. Similar results were obtained when identical samples of cells producing one of the four myc-tagged versions of Pex3p were used (data not shown). Remarkably, Pex3p was completely released from the membrane after incubation of a 30,000xg organellar pellet with 6 M urea. The integral membrane protein HpPex10p (Tan et al., 1995), used as control, was largely resistant to urea extraction under these conditions (Fig. 3B) as >75 % of the protein was observed in the pellet fraction. Catalase was also exclusively present in the

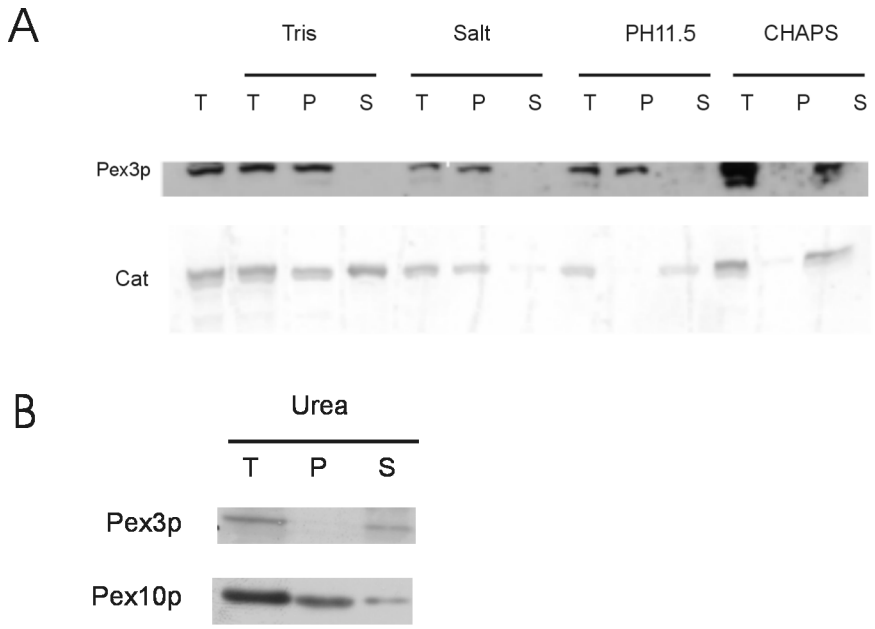

Fig. 3*. A.** *Extraction of 30.000xg organellar pellets prepared from wild type cells grown for 12 h in mineral media containing 0.5% methanol. For the extraction 150 µg of protein from an organellar pellet was used. This pellet was resuspended in 1.5 ml of the indicated solutions. Extractions were performed using:* ***TRIS*: 0.1 M TRIS-HCl, pH 8.0,* ***salt****: 1 M NaCl in 0.1 M TRIS-HCl, pH 8.0,* ***pH 11.5****: 0.1 M Na_2CO_3, pH 11.5, and* ***CHAPS:*** *1% CHAPS. After incubating for 30 min on ice, samples were subjected to centrifugation at 100,000xg at 4 ºC for 30 min.* ***T****: total amount of protein,* ***S****: supernatant fraction,* ***P****: pellet fraction. The results show that HpPex3p is virtually completely resistant to extraction at pH 11.5. The peroxisomal matrix protein catalase however, is already extensively solubilized after treatment with 0.1 M TRIS-HCl, pH 8.0.*
B. *Extraction similar organellar pellets using 6 M urea. Following incubation and centrifugation, the integral membrane protein Pex10p is predominantly present in the pellet fraction, while Pex3p is exclusively detected in the supernatant.*

supernatant, as expected (not shown). Taken together, our combined data lend support to the notion that HpPex3p does not span the membrane.

Localization of Pex3· GFP fusion proteins

WT *H. polymorpha* cells producing a Pex3· GFP fusion protein under control of the homologueous *PEX3* promoter were grown on methanol and analyzed by epifluorescence. As expected, the peroxisomes present in the cells displayed fluorescence that was observed as a rim surrounding the organelle. Surprisingly, the organelles also showed one strong fluorescent spot (Fig. 4A). Analysis of cells in the early stages of adaptation from glucose to methanol revealed that these spots ('patches') appeared very early, already in the initial stages of adaptation. This suggests that Pex3p might in part be concentrated on the peroxisomal membrane in protein complexes. Since the protein is produced under control of its endogenous promoter, this organization might facilitate the function of Pex3p. Alternatively, it may

represent an artifact due to the GFP fusion. However, the latter seems less likely since the hybrid Pex3p· GFP protein is normally functional and can fully complement the *pex3* deletion strain (data not shown).

In previous studies we have shown the role of the N-terminus of Pex3p in sorting of the protein to the peroxisomalmembrane (Baerends et al., 1996; Baerends et al., 2000). Here, we studied the localization of Pex3p lacking its N-terminal 50 amino acids fused to GFP (ΔN_{50}Pex3pGFP) using CLSM. The results, depicted in Fig. 4B, show that the

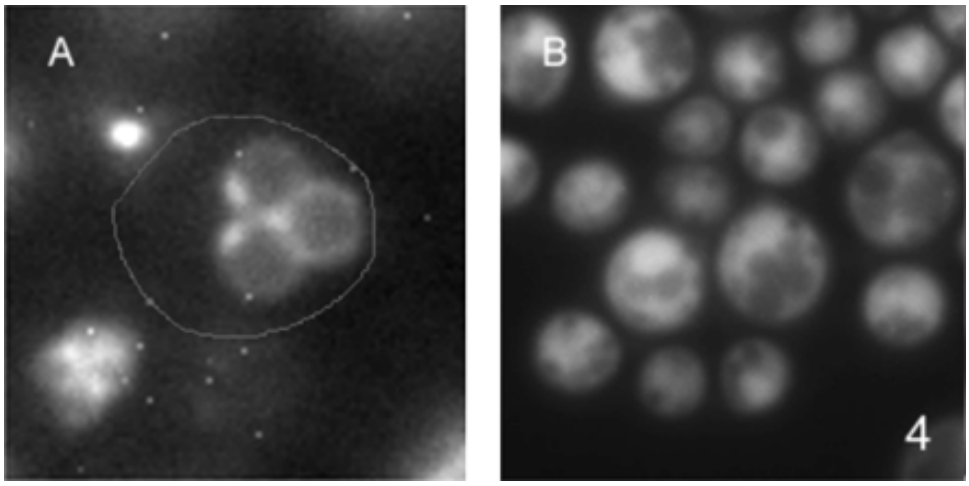

Fig. 4. ***A***. *Pex3p localization in* H. polymorpha *WT cells. Localization of the fusion protein is visualized by fluorescence microscopy. High magnification (10.000x) of WT* H. polymorpha *cells producing Pex3p.GFP. In this pictures solely three peroxisomes are visualised that display focal GFP fluorescent spots. The cell surface is indicated by the white line* ***B****. WT* H. polymorpha *cells producing ΔN_{50}.Pex3p.GFP; fluorescence is in the cytoplasm (x5000).*

fusion protein is cytosolic. This is in line with the described role of the N-terminus of Pex3p in sorting of this protein. The remaining part of the protein, consisting of aa 51-457, apparently does not contain sufficient information for sorting or binding of protein to the peroxisomal membrane.

Pre-embedding labeling experiments

Next, we decided to perform pre-embedding labeling experiments to gain further information on the HpPex3p topology. Fresh 30,000xg organellar pellets, prepared from methanol-grown cells of the various Pex3p-myc producing strains, were incubated with α-myc antibodies, thoroughly washed, incubated with GAM-gold, washed again and subsequently fixed with aldehyde/OsO_4 and embedded in Epon812 resin. The analysis of ultrathin sections, prepared from these samples, revealed that peroxisomes were covered with gold particles in all experiments, as shown in Fig. 5. The specificity

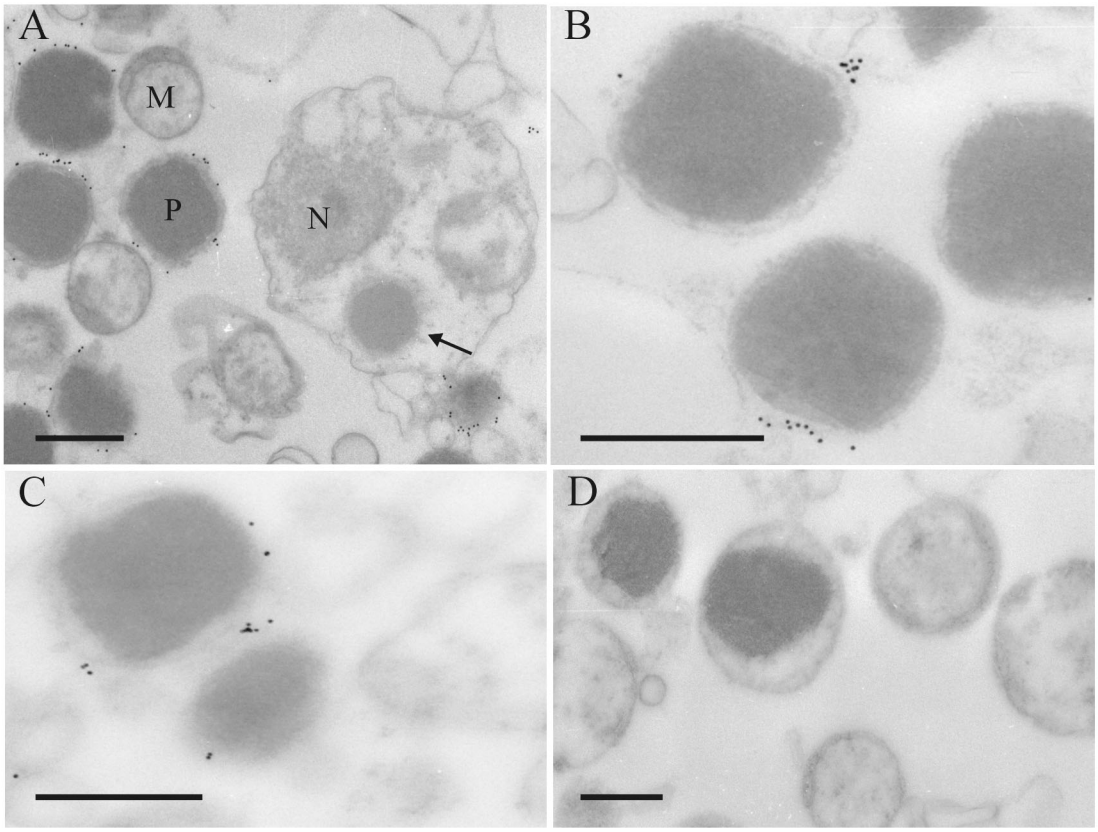

***Fig. 5**. Pre-embedding labeling of lysed protoplasts from cells grown for 12 h in batch cultures containing 0.5 % methanol. Monoclonal α-myc antibodies were used as primary antibodies. A. Specific labeling of the peroxisomal membrane in a preparation of lysed protoplasts from strain RBG1[P_{PEX3}.PEX3.102-111myc]). Mitochondria and peroxisomes in unlysed protoplasts are not labeled (arrow). B. Pre-embedding labeling of RBG1[P_{PEX3}.PEX3.N-myc], C. RBG1[P_{PEX3}.PEX3.C-myc], and D. wild type* H. polymorpha*. In all cases labeling is predominantly present at the surface of the peroxisomal membrane and frequently localized in clusters. In samples wild type control cells that do not produce myc tagged Pex3p, α-myc labeling is absent. (**M**: mitochondrion, **N**: nucleus, **P**: peroxisome). Bar indicates 0.5 μm.*

of the labeling was stressed by the finding that peroxisomes, which were present in unlysed protoplasts and thus were not accessible for the antibodies, invariably remained unstained (see Fig. 5A). Also mitochondria and other membranous structures, e.g. derived from the plasma membrane, which are present in the samples invariably showed no labeling. In all samples the labeling was frequently observed to be concentrated on small areas of the peroxisomal membrane, comparable to the focal distribution of Pex3p· GFP visible in the fluorescence images (see Fig. 4). Remarkably, the frequency of the gold label was highest on peroxisomes from RBG1[P_{PEX3}.*PEX3· C-myc*] cells (Fig. 5C) and was relatively low on organelles of RBG1[P_{PEX3}.*PEX3· N-myc*] cells (Fig 5B). The significantly lower labeling frequencies in case of the N-terminal myc might reflect a reduced accessibility of the N-terminal part of Pex3p for the antibodies, for instance caused by protein-protein interactions occurring at this part of the protein. However, we cannot exclude the possibility that

folding of the protein itself causes these apparent differences in accessibility of the different regions of Pex3p.

In WT control samples α-myc specific labeling was never observed (see Fig. 5D). However, using α-Pex3p antibodies and GAR-gold, the isolated organelles displayed labeling again (data not shown).

Freeze etch analysis

A remarkable characteristic of peroxisomes of *H. polymorpha* is that the fracture faces of their surrounding membrane are largely smooth (Sulter et al., 1990); not shown, compare Fig. 6C), a phenomenon that is indicative for the low abundance of large integral membrane proteins. This architecture is remarkable, since the organelles have multiple functions that are expected to require active membrane transport. Therefore we decided to investigate the architecture of these membranes in more detail. First, we analyzed organelles in cells that overproduced either *H. polymorpha* Pex10p, (Tan et al., 1995) or *Candida boidinii* PMP47 (Goodman et al., 1986), proteins that are known to span the peroxisomal membrane in *H. polymorpha* (Sulter et al., 1993). Overexpression of both proteins had indeed occurred, as was evident from Western blots (data not shown). After freeze etching, the fracture faces of the peroxisomal membranes clearly show the presence of particles of largely uniform size (Fig. 6A, B). We interpreted this to be the result of the overproduction of the membrane proteins and therefore these data are in line with the finding that integral membrane proteins, if present, can be resolved by freeze fracturing (Eskandari et al., 1998). Subsequently we analyzed cells, which overproduced full-length Pex3p. In these cells the replicas showed again the smooth fracture faces typical for WT *H. polymorpha* (Fig. 6C). However, the analysis of isolated peroxisomes of which the surface was visualized after deep etching, again resolved particles that were associated with the cytosolic face of the membrane (Fig. 6D). These combined data lend support to two important implications. Firstly, peroxisomes of *H. polymorpha* lack abundant large integral membrane proteins as compared to e.g. vacuoles and mitochondria; however, proteins are observed to be associated with the surface of the organelles. Secondly, we found no evidence, using freeze-fracturing techniques, that Pex3p has multiple membrane spans.

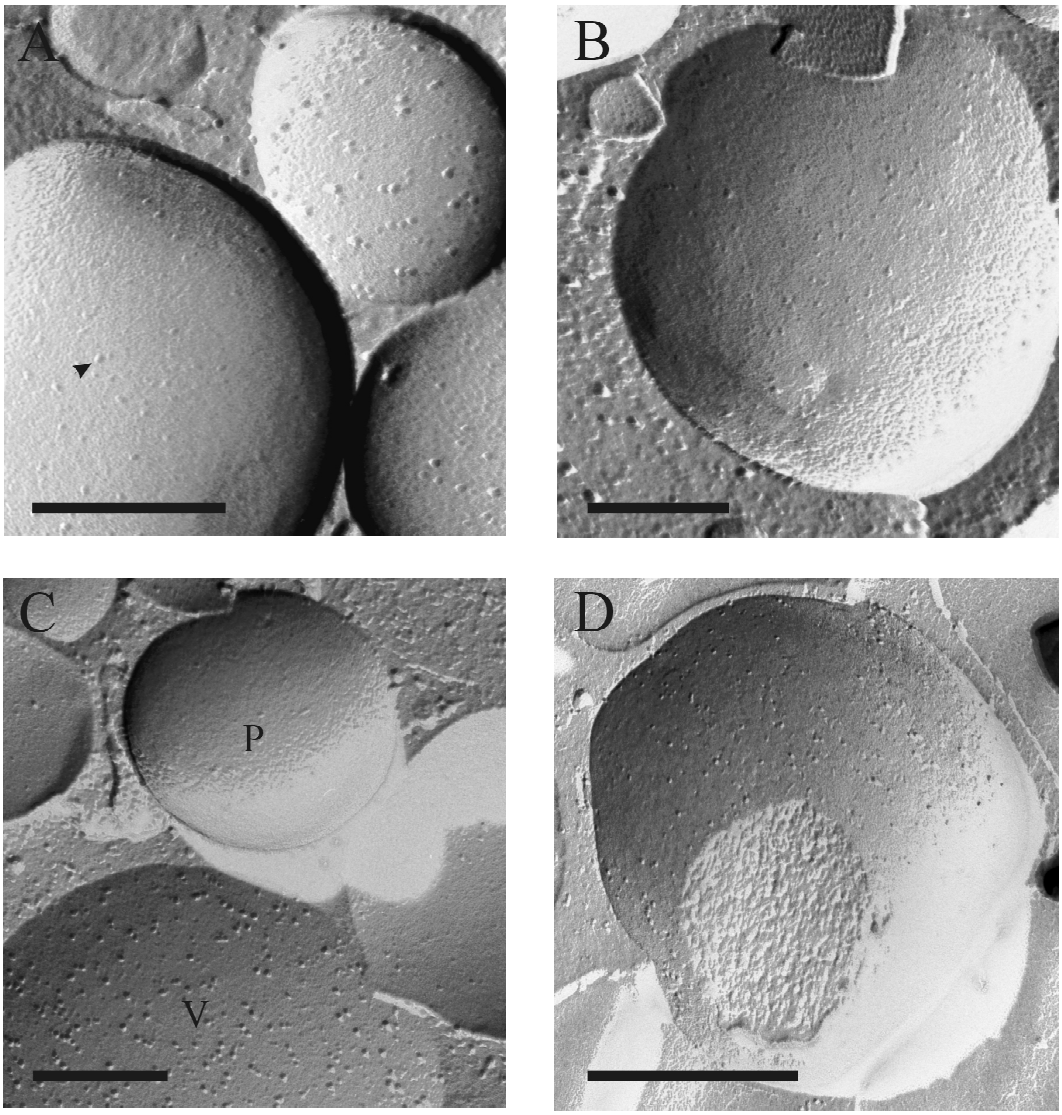

Fig. 6. *Freeze etch analysis of* H. polymorpha *peroxisomal membranes in wild type cells grown on methanol. A.* H. polymorpha *CT103, overexpressing HpPex10p. The particles (arrowhead) on the peroxisomal membranes indicate the presence of integral membrane proteins.*
B. WT::YEpMOX47, overproducing CbPMP47, also showing a relative dense particulate architecture. C. WT::P_{AOX}PEX3^{1x} *cells overproducing HpPex3p. The smooth surface of peroxisomes is apparent. D. Characteristic overview of a peroxisome, purified from WT*
H. polymorpha *cells, grown on methanol. After fixation in aldehyde solution the samples were subjected to freeze-etching. After fracturing, the surface of the organelle, recognized by crystalline matrix (*), is visualised after deep etching showing the presence of protein particles at the cytosolic surface of the peroxisomal membrane. (**V**: vacuole, **P**: peroxisome). Bar indicates 0.25 µm.*

Discussion

Hansenula polymorpha Pex3p (Baerends et al., 1996) is a component of the peroxisomal membrane and homologues have been described of various organisms (Wiemer et al., 1996; Kammerer et al., 1998; Höhfeld et al., 1991; Soukupova et al., 1999). In the initial description of the bakers yeast protein (ScPex3p, (Höhfeld et al., 1991)) it was proposed that the protein spans the membrane at the N-terminus (aa 18-39) in conjunction with a membrane-associated region at aa 135-153. This topology has been questioned for RnPex3p (Ghaedi et al., 2000), and other modes of insertion have been proposed. In this paper we provide various lines of evidence that *H. polymorpha* Pex3p is tightly associated to the cytosolic face of the organellar membrane, without spanning it. In favor of this orientation are the results obtained by the analysis of the protease sensitivity of HpPex3p, using intact organellar fractions. In numerous experiments Pex3p was invariably completely removed in the presence of externally added proteases. This was also the case with myc-tagged versions in which tags have been introduced at selected positions in the protein. The positions of these tags were based on the location of the three predicted hydrophobic regions in the protein (using several algorithms) that could represent membrane spans. These stretches are located at the aa positions 16-36, 159-179, and 366-387. The myc tags were introduced at positions in between the three possible membrane-spanning domains and at the extreme N- and C-termini of the protein. All the constructed, tagged versions of the protein were shown to fully complement the *pex3* phenotype. Both the pre-embedding labeling experiments and the protease protection assays using the hybrid Pex3p proteins gave similar results in that all introduced myc tags were accessible at the cytosolic face of the peroxisomal membrane.

However, as shown before in biochemical extraction experiments, HpPex3p (Baerends et al., 1996), but also ScPex3p (Höhfeld et al., 1991), displays properties of an integral membrane protein as it is resistant towards carbonate extraction. Several examples are however available, also from peroxisomal proteins, that suggest that the carbonate treatment does not always give an unequivocal discrimination in case of integral membrane proteins. For instance, ScPex13p and the peroxisomal ABC transporter PAT1, both recognized integral membrane proteins, show bimodal distribution patterns upon carbonate treatment (Elgersma et al., 1996). It can be envisaged that the opposite may also occur: tightly associated proteins, which do not span the membrane, may -in part- be resistant to carbonate treatment. Interestingly, Johnson *et al*. raised the same issue in their discussion of the location and topology of *P. pastoris* Pex14p (Johnson et al., 2001). They

conclude that although PpPex14p behaves like an integral membrane protein upon carbonate treatment, it does not contain any membrane spanning regions, contrary to results published of other Pex14p homologues (e.g. human (Will et al., 1999), and *H. polymorpha* Pex14p (Komori et al., 1997)). Therefore they conclude that tight association with other integral membrane proteins probably causes the strong interaction of Pex14p with the peroxisomal membrane in *P. pastoris*.

Freeze fracture experiments on WT peroxisomes suggest that peroxisomes are devoid of high quantities of large integral membrane proteins. That this assumption is indeed true was clear from experiments on cells that overproduce either of two proteins known to span the membrane, CbPMP47 or HpPex10p. Overproduction of both full-length Pex3p and Pex3p$_{[1-50]}$GFP did not enhance the particle numbers of the fracture faces of the peroxisomal membrane, suggesting that HpPex3p does not contain membrane spans. This view was strengthened by the data of the urea extractions.

Taken together, our data support the assumption that HpPex3p is very tightly associated to the cytosolic face of the peroxisomal membrane, where it may be present in protein clusters, judged from the fluorescence data. Most probably, these clusters represent homomeric or heteromeric protein complexes at the peroxisomal membrane. We are currently investigating high molecular weight protein complexes in the peroxisomal membrane of *H. polymorpha* to identify the constituents of such complexes (Lutz and van der Klei, unpublished results). These studies will also establish whether HpPex3p is a component of such complexes. One possible clue to the presence of Pex3p in clusters may be related to the fact that the ratio between the increase of Pex3p levels (approximately 6-fold, (Baerends et al., 1996)) under methylotrophic growth conditions and the increase in peroxisomal membrane surface (>1,000-fold) is very low. Thus, to maintain functional quantities of Pex3p, a focal concentration may be required to avoid ineffective dilution of the protein over the membrane. This conformation may also, at least in part, explain the tight association of the protein to the membrane, based on biochemical criteria. Moreover, the tight association might be related to the possibility that HpPex3p has one or more proteinaceous binding partners at the peroxisomal surface, which tightly anchor the protein to the membrane and/or other proteins. The N-terminus of Pex3p would be a likely part to interact in such a complex, since the labeling intensity of the N-terminally tagged versions is lower than that for C-terminally tagged Pex3p in immunocytochemistry. Interestingly, Soukupova *et al*. (Soukupova et al., 1999) made similar observations when studying the topology of HsPex3p using an approach

based on immunofluorescence. They concluded that the N-terminus of HsPex3p protruded into the peroxisomal matrix, although they could not exclude other modes of protection of the N-terminus. The involvement of the N-terminus of HsPex3p in binding to other proteins could be an alternative explanation for this phenomenon.

The importance of the N-terminus of Pex3p for its localization was also shown in our approach to study the targeting of ΔN_{50}· Pex3p· GFP in WT cells. The cytosolic localization we observed for this truncated fusion protein clearly indicates that the N-terminal 50 aa are critically important for targeting of Pex3p. Either the targeting itself, or alternatively, the association to the membrane is disturbed in this situation. However, it could not be determined whether folding of the truncated protein was disturbed. Abnormal folding could also account for the loss of binding capability of ΔN_{50}· Pex3p.

In *P. pastoris* it was shown that the N-terminal 40 amino acids of Pex3p fused to GFP were targeted to, and tightly associated with, the peroxisomal membrane (Wiemer et al., 1996), although a putative transmembrane domain is not observed in this fusion protein. This also suggests that the N-terminus of PpPex3p tightly binds to the peroxisomal membrane without the involvement of membrane spanning regions. Our finding that both N- and C-termini of HpPex3p are cytosolic, resemble the results published by Ghaedi *et al*. for RnPex3p (Ghaedi et al., 2000).

The binding partners of Pex3p identified to date are Pex14p, (Snyder et al., 1999) and Pex19p (Götte et al., 1998), (Hettema et al., 2000). Pex14p is a peroxisomal membrane protein and is thought to be a component of the docking site of the cytosolic receptors, Pex5p and Pex7p, of peroxisomal PTS1 or PTS2 matrix proteins (Albertini et al., 1997). Pex19p is a farnesylated integral membrane protein that interacts with multiple peroxisomal membrane proteins. We have not yet been able to determine whether Pex14p or Pex19p are involved in binding of Pex3p to the peroxisomal membrane of *H. polymorpha*.

When the transmembrane predictions of yeast and human Pex3p proteins are compared, striking differences are apparent (Kammerer et al., 1998). Consequently, Pex3p may show different topologies in these species although the proteins are thought to be functional homologues. The latter may not only be hypothetical since heterologous complementation of the *H. polymorpha pex3* mutant by the *S. cerevisiae PEX3* gene product has been demonstrated (Kiel et al., 1995), although these proteins show a sequence homology of only 30%. Rather low identities between proteins, which are thought to be functional homologues, seem to be a typical characteristic of peroxins. In this context, the results we obtained using the

MEMGEN algorithm (Lolkema and Slotboom, 1998) are noteworthy. MEMGEN performs hydropathy profile alignments, and when provided with the sequences of *H. polymorpha*, *S. cerevisiae*, *H. sapiens*, and *R. norvegicus* Pex3p, the results depicted in Fig. 7 are obtained. No obvious membrane-spanning regions with sufficient length and hydrophobicity become apparent when these sequences are aligned. Therefore, on the basis of a hydropathy profile alignment, no clear consensus can be found with respect to the presence of putative membrane spans in the Pex3p homologues.

Until now, an in depth analysis of the topology of Pex3p has not been performed in any organism. Since comparison of sequence data and transmembrane predictions show quite dissimilar results for the homologues from different species, it remains unclear whether these proteins actually share the same topology. The proposed mode for membrane attachment of HpPex3p therefore might not reflect the situation in other species studied so far. Further understanding of the role of this peroxin in peroxisome biogenesis and maintenance, will depend on identifying its binding partners and thus gaining insight into the molecular mechanisms of its activity.

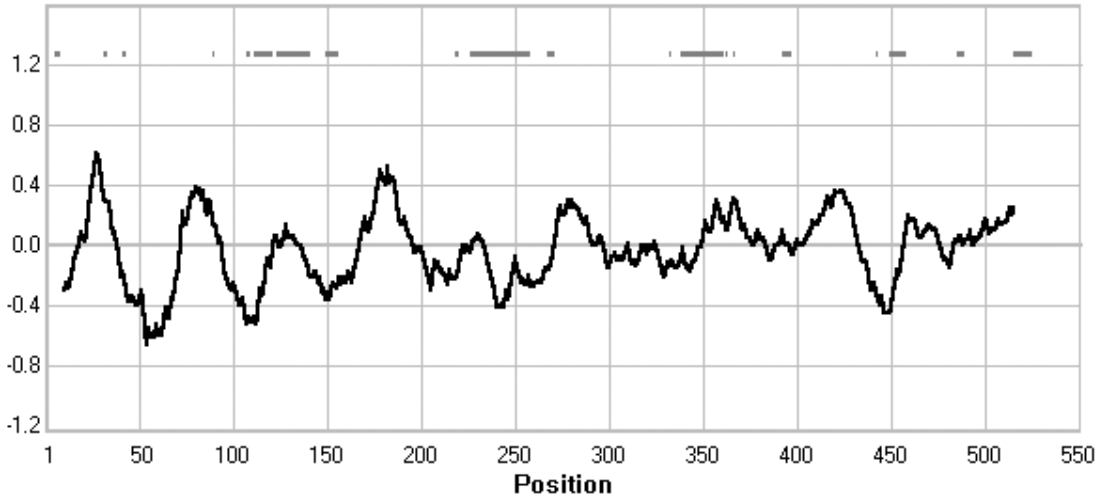

Fig. 7*. Hydropathy profile alignment of Pex3p using MEMGEN. The amino acid sequences of Pex3p from* H. polymorpha, S. cerevisiae, Homo sapiens*, and* R. norvegicus *were used. The horizontal axis represents amino acid positions of the aligned sequences, the vertical axis represents hydropathy. Grey bars indicate gaps introduced into the aligned sequences.*

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Chapter 4

Normal peroxisome development from vesicles induced by truncated *Hansenula polymorpha* Pex3p

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Abstract

We show that the synthesis of the N-terminal 50 amino acids of Pex3p [$Pex3p_{[1-50]}$] in *Hansenula polymorpha pex3* cells is associated with the formation of vesicular membrane structures. Biochemical and ultrastructural findings suggest that the nuclear membrane is the donor membrane compartment of these vesicles. These structures also contain Pex14p and can develop into functional peroxisomes after subsequent re-introduction of the full-length Pex3p protein. We discuss the significance of this finding in relation to peroxisome re-introduction, e.g. in case peroxisomes are lost due to failure in inheritance.

Introduction

Peroxisomes are remarkable among the various classes of cell organelles in that their function and abundance varies dependent on the organism, the environmental conditions and the physiological state of the cell. Yeasts are favorable model organisms to study peroxisome biogenesis because of a number of properties. Firstly, peroxisome proliferation can be strictly regulated by growth conditions, ranging from one small organelle when cells are grown in rich media containing glucose, to over 20 during growth of cells on oleate (*Saccharomyces cerevisiae, Yarrowia lipolytica* and *Pichia pastoris*) or methanol (*Hansenula polymorpha, P. pastoris*) (Veenhuis, 1992; Kunau, 1998). Under these conditions, peroxisomes house the key enzymes involved in the metabolism of these carbon sources. Secondly, peroxisomes are not essential for yeast cell viability when they are grown under peroxisome-repressing conditions in rich media. This feature has led to the isolation of various yeast peroxisome assembly (*pex*-) deficient mutants that are unable to utilise either oleate or methanol for growth (Erdmann et al., 1997; Subramani, 1998; Hettema et al., 1999). With few exceptions (see below), these *pex* mutant cells still contain peroxisomal membrane remnants (‘ghosts”). Through genetic complementation, 23 *PEX* genes have currently been characterised (Hettema et al., 1999). Database searches using the yeast genes have presently revealed 13 human orthologes of yeast *PEX* genes. Mutations in 11 of these human *PEX* genes have been characterised and shown to be the molecular basis for inherited peroxisome biogenesis disorders, including Zellweger Syndrome, neonatal adrenoleukodystrophy, infantile Refsum’s disease and rhizomelic chondrodysplasia punctata (Braverman et al., 1995; Wanders, 1999).

The analysis of the *PEX* genes and the peroxins they encode has predominantly shed light on the molecular components involved in peroxisomal matrix protein

import (reviewed by (Subramani, 1998; Hettema et al., 1999). Relatively less is known of the biogenesis of the peroxisomal membrane and the sorting mechanisms of the proteins it contains. Earlier work showed that peroxisomal membrane proteins, like peroxisomal matrix proteins, are synthesised on free polysomes in the cytosol and are post-translationally transported to the peroxisome (Fujiki et al., 1984). Together with morphological studies on peroxisome proliferation, these data have led to the hypothesis that peroxisomes develop by fission from pre-existing ones (Lazarow and Fujiki, 1985). Recent research, however, suggested that some peroxisomal membrane proteins may travel via the ER to the peroxisome and that vesicle budding and fusion processes may be involved in peroxisome growth and maturation (Titorenko and Rachubinski, 1998; Faber et al., 1998; Mullen et al., 1999; Titorenko et al., 2000).

Notably, the prevailing model of budding from pre-existing peroxisomes cannot explain the re-assembly of peroxisomes in mutant cells lacking any peroxisomal remnants (yeast and human *pex3, H. polymorpha per13-6^{ts},* human *pex16*, and *S. cerevisiae* and human *pex19*) upon functional complementation of such cells (Höhfeld et al., 1991; Waterham et al., 1993; Baerends et al., 1996; Wiemer et al., 1996; Götte et al., 1998; Matsuzono et al., 1999; South and Gould, 1999; South et al., 2000). Several recent studies have assessed the question of the origin of the peroxisomal membrane upon re-appearance of the organelles in *pex*-mutants lacking peroxisomal membranes. We studied this process in a temperature-sensitive (ts) *pex* mutant of the yeast *H. polymorpha* (Waterham et al., 1993). Peroxisomal membrane remnants were undetectable in these cells when grown at restrictive temperatures (43^{o}C). Shifting these cells to permissive conditions (37^{o}C) led to a rapid (within 30 min) reappearance of peroxisomes. Concurrently, South and Gould (1999), Matsuzono *et al*. (1999) and South *et al*. (2000) studied the reappearance of peroxisomes in human cells defective for *PEX16, PEX19* and *PEX3*, respectively. Peroxisomal remnants were absent in these cells also, but intact organelles were assembled upon re-introduction of the complementing gene. As suggested before by Waterham *et al.* (1993), these studies revealed that peroxisomes do not necessarily derive from pre-existing ones, but failed to identify the alternative origin.

Therefore, we set out to study the reappearance of peroxisomes in *H. polymorpha pex3* cells in detail upon re-introduction of full-length Pex3p, a peroxisomal membrane protein, and hybrid proteins consisting of N-terminal fragments of Pex3p and either GFP or β-lactamase. Here we show that the N-terminal 50 amino acids of Pex3p induce the formation of vesicles in the vicinity of the nuclear membrane.

These Pex3p$_{[1-50]}$vesicles are the specific target for peroxisome development after subsequent synthesis of full-length Pex3p.

Materials and methods

Strains and growth conditions

H. polymorpha NCYC495 (*leu1.1*) and derivatives of this strain (listed in Table I) were grown at 37^{o}C in batch cultures in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or in mineral medium (van Dijken et al., 1976) containing either 0.5% (w/v) glucose, 0.5% (v/v) methanol or a mixture of 0.1% (v/v) glycerol and 0.5% methanol as carbon and energy source in combination with 0.25% (w/v) ammonium sulphate or 0.25% (w/v) ethylamine as sole nitrogen sources. For growth on solid media, 0.67% (w/v) yeast nitrogen base was used supplemented with 1% (w/v) glucose, and 2% (w/v) agar. When required, leucine was added to the media to a final concentration of 30 mg/l.

Table I. *H. polymorpha* strains used in this study.

Strain	Relevant genotype	Reference
NCYC495	*H. polymorpha* WT, *leu1.1* derivative	(Gleeson and Sudbery, 1988)
RBG1	*pex3::URA3*, *leu1.1*	(Baerends et al., 2000)
RBG17	*pex3*::P$_{AOX}$-*PEX3*^{1x}	This study
HF75	NCYC495::P$_{AOX}$-*PEX3*$_{[1-50]}$*GFP*^{2x}	This study
HF78	*pex3*::P$_{AOX}$-*PEX3*$_{[1-50]}$*GFP*^{2x}	This study
HF245	*pex3*::P$_{AOX}$-*PEX3*^{1x}::P$_{AMO}$-*PEX3*$_{[1-50]}$*GFP*^{1x}	This study
HF290	*pex3*::P$_{AOX}$ -*PEX3*$_{[1-50]}$*β-lactamase*^{3x}::P$_{AMO}$-*BiP*$_{[1-30]}$*GFP*^{2x}	This study
HF305	*pex3*::P$_{PEX3}$-*PEX3*$_{[1-50]}$*GFP*	This study

Superscript ($^{1x,2x \text{ or } 3x}$) refers to the number of expression cassettes integrated in the genome of the transformed strain.

Molecular biological techniques

Escherichia coli DH5α and XL1blue were used for the propagation and amplification of plasmid DNA. Recombinant DNA procedures (enzyme digestion, cloning, plasmid isolation, PCR and Southern blotting) were performed essentially as described by (Sambrook et al., 1989). Transformation of *H. polymorpha* strains and site-specific integration of single and multiple copies of plasmid DNA in the genomic *AOX*- or

AMO-locus was performed as described (Faber et al., 1994a; Faber et al., 1994b; Baerends et al., 1996).

Plasmid constructions

Expression plasmids pHIPX4-PEX3 (Kiel et al., 1995) and pHIPX4-PEX3$_{[1-50]}$GFP (pFEM75) (Baerends et al., 2000) have been detailed before. pHIPX6-PEX3$_{[1-50]}$GFP (pFEM64) was obtained by inserting a 0.9 kb *BamHI-SalI* from pFEM75 into *Bam*HI-*Sal*I digested pHIPX6 (Kiel et al., 1995), an *H. polymorpha* expression plasmid containing the *PEX3* promoter element. For co-expression of reporter genes, novel *H. polymorpha* expression vectors were constructed based on the dominant zeocin resistance gene. Vector pHIPZ4, containing the *H. polymorpha* alcohol oxidase promoter (P_{AOX}) for heterologous expression has recently been described (Salomons et al., 2000). pHIPZ5, containing the *H. polymorpha* amine oxidase promoter (P_{AMO}) was constructed by inserting an 1.0-kb *Not*I-*Bam*HI DNA fragment from pHIPX5 (Kiel et al., 1995) containing the P_{AMO} into *Not*I-*Bam*HI digested pHIPZ4. pHIPZ4-PEX3$_{[1-50]}$β-lactamase (pFEM201) was constructed as follows: the β-lactamase reporter gene was subcloned in pBluescript II SK$^+$ (Stratagene, La Jolla, CA) as a 0.8-kb *Eco*RI-*Hin*dIII fragment from pGF154 (Faber et al., 1995) and subsequently inserted as a *Sma*I-*Sal*I fragment into pBS-*PEX3* (Baerends et al., 2000) digested with *Nco*I (Klenow fill-in)-*Sal*I, resulting in pFEM32. Subsequently, a 0.15-kb PCR fragment obtained using primers per9ATG (Kiel et al., 1995) and pex3-FT2 (Baerends et al., 2000) was digested with *Bgl*II and *Nco*I and inserted in *Bgl*II-*Nco*I digested pFEM32, resulting in pFEM30. The PEX3$_{[1-50]}$β-lactamase hybrid gene was excised from pFEM30 by *Bam*HI-*Sal*I digestion and inserted into *Bam*HI-*Sal*I digested pHIPZ4, resulting in pFEM201. pHIPZ5-PEX3$_{[1-50]}$GFP (pFEM167) was constructed by inserting a 0.9-kb *Bam*HI-*Sma*I DNA fragment from pFEM 75 (Baerends et al., 2000) into *Bam*HI-*Sma*I digested pHIPZ5. pHIPX5-*BiP$_{[1-30]}$GFP* (pFEM76) was constructed as follows: Using PCR and primers KN18 (5' CCCAAGCTT*GGATCC* ATG TTA ACT TTC AAT AAG TC 3') and KN19 (5' GGGAAGCTT*AGATCT* AAA CTG CTG TGT TGT TAG TGG G 3') a *Bam*HI site was introduced upstream the startcodon, and a *Bgl*II site downstream codon 30 (Phe) of the *H. polymorpha KAR2* gene. In addition, using PCR and primers KN14 (5' CCCCTC GAG AAC CTG TAC TTC CAG TCG *AGA TCT* GTG AGC AAG GGC GAG GAG C 3') and eGFP-SalI (Baerends et al., 2000) a *Bgl*II site was introduced upstream codon 2 (Val), and a *Sal*I site downstream the stopcodon of the eGFP gene (Clonetech, Germany). The *Bgl*II sites were used to fuse the BiP$_{[1-30]}$ and the eGFP genes. The flanking *Bam*HI and the *Sal*I

sites were used to clone the hybrid gene downstream the amine oxidase promoter in expression vector pHIPX5.

Biochemical methods

Crude extracts of *H. polymorpha* were prepared according to (Baerends et al., 2000). SDS-PAGE (Laemmli, 1970) and Western blot analysis (Kyhse-Andersen, 1984) were performed as described; blots were decorated using specific antibodies against various *H. polymorpha* proteins. The antibodies against *S. cerevisiae* Sec63p, which cross-react with the *H. polymorpha* Sec63p orthologue, were a gift from Dr. R. Schekman, Berkeley, USA. The antibodies against GFP, and *S. cerevisiae* cytosolic ADH, which cross react with the *H. polymorpha* ADH orthologue, were a gift from Dr. W.-H. Kunau, Bochum, Germany. Goat anti-rabbit alkaline phosphatase and goat anti-rabbit horse radish peroxidase (Roche Molecular Biochemical, Almere, The Netherlands) were used as secondary antibodies which were detected by bromo-chloroindolyl phosphate/Nitro blue tetrazolium (Roche Molecular Biochemical, Almere, The Netherlands) or ECL (Amersham, Arlington Heights, IL) according to manufacturers' protocols. Enzyme activity of cytochrome c oxidase was determined as described (Douma et al., 1985). Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as standard. Cell fractionation experiments were performed as detailed by van der Klei *et al.* (van der Klei et al., 1998). In addition to the standard sucrose density gradients routinely used to purify peroxisomes from WT *H. polymorpha*, we used a modified gradient (consisting of 4 ml 65%, 4 ml 50%, 4 ml 46%, 8 ml 40% 4 ml 35% and 4 ml 30% (w/v)-sucrose in buffer B (5 mM MES, 0.1 mM EDTA, 1 mM KCl, pH 5.5) loaded with an organellar pellet resuspended in 5 ml homogenisation buffer (5 mM MES, 0.1 mM EDTA, 1 mM KCl, 1.2 M sorbitol, pH 5.5) and centrifuged for 3 h at 33,000 x g.

Microscopy

Fluorescent microscopy to localise hybrid proteins containing GFP was carried out according to Baerends et al., (2000).

Whole cells and organellar fractions were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Douma et al., 1987; Waterham et al., 1994). Immunolabelling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against various *H. polymorpha* proteins, GFP and β-lactamase and gold-conjugated goat anti-rabbit (GAR-gold) antibodies

according to the instructions of the manufacturer (Amersham Corp. Arlington Heights, IL). Double immunocytochemical labelling using two polyclonal antisera and different sized gold particles (5-nm and 15-nm GAR-gold) was performed according to Bendayan (1982).

Results

Synthesis of Pex3p$_{[1-50]}$ causes vesicle formation in pex3 cells

Similar to *pex3* cells from other organisms, *H. polymorpha pex3* cells grown in batch cultures on methanol, do not contain detectable peroxisomal membrane remnants and proteins normally residing in the peroxisomal matrix, accumulate in the cytosol. However, normal peroxisomes rapidly re-appear in such cells upon re-introduction of the complementing *PEX3* gene (Baerends et al., 1996). In an attempt to shed light on the origin of these organelles, we introduced hybrid genes encoding N-terminal fragments of Pex3p (containing its putative peroxisomal targeting signal) and a reporter gene (GFP or β-lactamase) in *pex3* cells and determined the subcellular location of the gene products. *pex3* cells producing the Pex3p$_{[1-50]}$GFP hybrid protein (strain HF78) contained one or few bright fluorescent spots (Fig. 1B right panel); in WT controls (strain HF75) this protein was efficiently targeted to peroxisomes (Fig 1A, right panel; (Baerends et al., 2000). Electron microscopy showed that HF78 cells contained a cluster of small vesicular structures in close proximity to the nucleus (Fig. 1C). These structures were never observed in the *pex3* parental strain (not shown, cf. (Baerends et al., 1996) or in WT cells. These membrane structures were the subcellular site of the Pex3p$_{[1-50]}$GFP hybrid protein as was shown by immunocytochemistry, using α-GFP antibodies (Fig. 1D). The presence of these membranes was not the result of the overproduction of the hybrid protein. Similar patterns of fluorescence (Fig 1C inset) and membrane vesicles (not shown) were observed when the Pex3p$_{[1-50]}$GFP protein was produced by the *PEX3*-promoter element, though less abundantly. These data show that synthesis of the 50 N-terminal amino acids of Pex3p fused to a reporter protein, induces proliferation of membranous structures. From here, we refer to these membrane structures as Pex3p$_{[1-50]}$-vesicles.

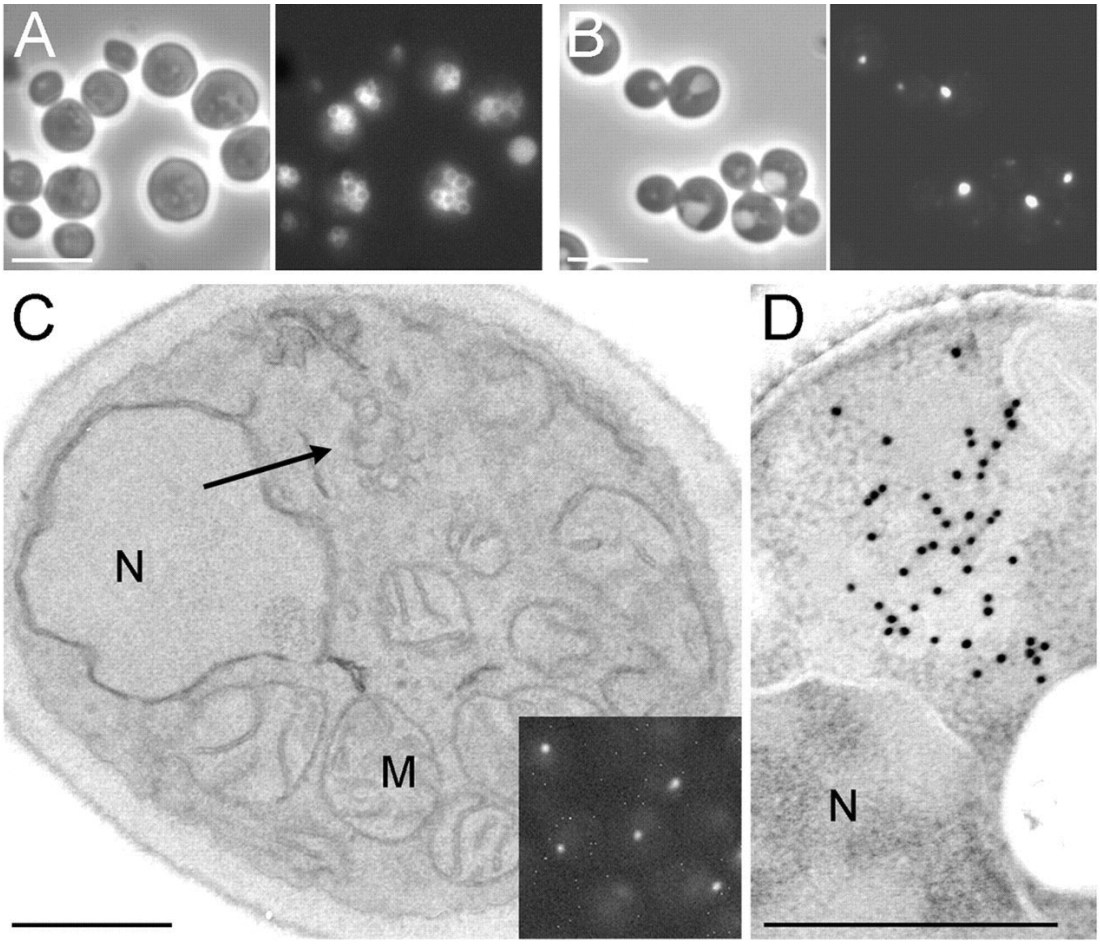

Fig. 1. *Synthesis of Pex3p$_{[1-50]}$GFP causes vesicle formation in pex3 cells. Cells were grown in glycerol/methanol-medium for 16 h. A,B, fluorescence microscopic analysis of HF75 (WT::P$_{AOX}$-PEX3$_{[1-50]}$GFP) cells (A) and HF78 (*pex3::P$_{AOX}$-PEX3$_{[1-50]}$GFP*) cells (B). Left panels, bright field images; right panels fluorescent images. C,D, electron microscopic analysis of glycerol/methanol-grown HF78 cells. Membrane/vesicle clusters are observed in these cells (C, arrow), which were not found in WT control cells (not shown). The inset shows a fluorescent image of HF305 cells, synthesising Pex3$_{[1-50]}$-GFP by the PEX3-promoter, grown in methanol-containing media. The Pex3p$_{[1-50]}$GFP hybrid protein is present in these membrane clusters as shown by immunolabelling using GFP-specific antibodies (D). Abbreviations: M, mitochondrion, N, nucleus. Bar = 5 µm (A,B) and 0.5 µm (C,D).*

Pex3p$_{[1-50]}$-vesicles display peroxisomal characteristics.

To determine the nature of the Pex3p$_{[1-50]}$-vesicles, we set out to purify them, using GFP as a marker protein. The conventional biochemical procedure for the isolation of intact peroxisomes from *H. polymorpha* WT cells [homogenisation of protoplasts followed by sucrose-density gradient centrifugation of a post-nuclear supernatant (PNS)] applied on HF78 cells resulted in a distinct localisation of Pex3p$_{[1-50]}$GFP at densities of approximately 30-40% sucrose, colocalising with ER and mitochondrial marker proteins (Fig. 2). To separate the Pex3p$_{[1-50]}$-vesicles from other cell constituents, we adjusted the sucrose density profile of the gradient to obtain a better

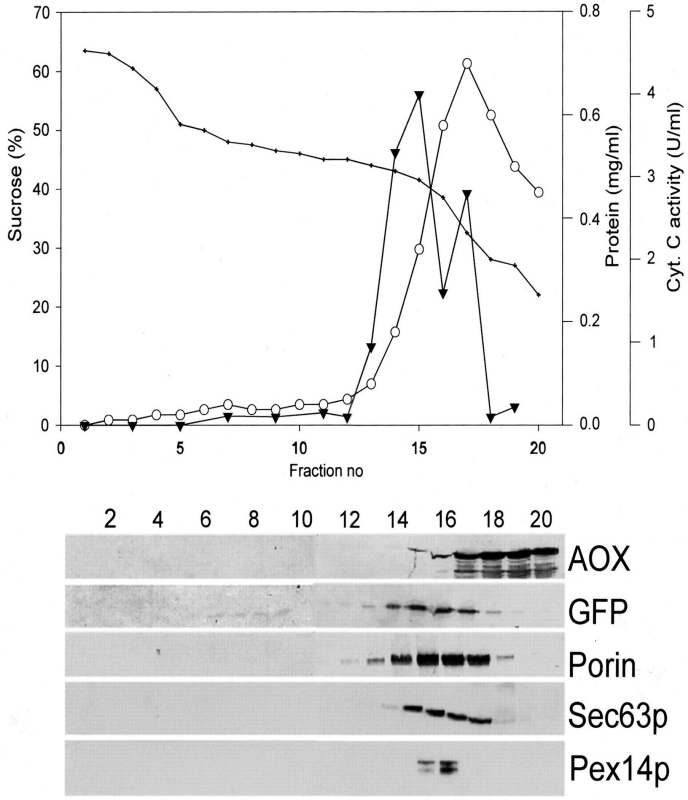

Fig. 2. *Pex3p$_{[1-50]}$-vesicles migrate to low-density fractions after sucrose density gradient centrifugation. Sucrose density centrifugation of a post-nuclear supernatant (PNS) obtained from glycerol/methanol-grown HF78 cells. Individual fractions were analysed for sucrose density (+), protein concentration (**O**) and mitochondrial cytochrome c oxidase activity (▼). Western blot analysis was used to determine the location of alcohol oxidase (AOX), Pex3p$_{[1-50]}$GFP (GFP), mitochondrial porin, endoplasic reticulum Sec63p and peroxin Pex14p. All membrane-bound marker proteins co-localise in the top fractions of the gradient.*

separation at sucrose concentrations of 30% to 40% and loaded the gradient with an organellar fraction instead of a PNS. Fig. 3 shows that a clear separation was achieved between the Pex3p$_{[1-50]}$-vesicles (peak fractions at 32-36% sucrose) and the mitochondria (peak fractions at 36-42% sucrose). Comparison of the fractions most enriched for Pex3p$_{[1-50]}$GFP, mitochondria and ER marker proteins (peak fractions at 38-41% sucrose) revealed that Pex14p, a peroxisomal membrane protein that is mislocalised to mitochondria in *pex3* cells (Baerends et al., 2000), now co-localised with Pex3p$_{[1-50]}$GFP (Fig. 3B). This finding was further substantiated by immunocytochemical analysis of the organellar fraction (Fig. 4A,B) and whole cells (Fig. 6E, see below). The Pex3p$_{[1-50]}$-vesicles were readily identifiable in the organellar fraction by immunocytochemistry, using specific antibodies against GFP, and were clearly distinct from other structures in these fractions, such as mitochondria and plasma membrane vesicles (Fig. 4A). Clearly, α-Pex14p-dependent specific labelling was only found on the Pex3p$_{[1-50]}$-vesicles (Fig. 4B). In addition, co-localisation of Pex3p$_{[1-50]}$β-lactamase and endogenous Pex14p on the Pex3p$_{[1-50]}$-vesicles was observed in double-labelling experiments performed on ultrathin sections of *pex3* cells producing Pex3p$_{[1-50]}$β-lactamase (Fig. 6E).

To further analyse the possible peroxisomal nature of the Pex3p$_{[1-50]}$-vesicles, we investigated whether these structures were susceptible to selective degradation, comparable to WT peroxisomes (Veenhuis et al., 1983) and peroxisomal remnants

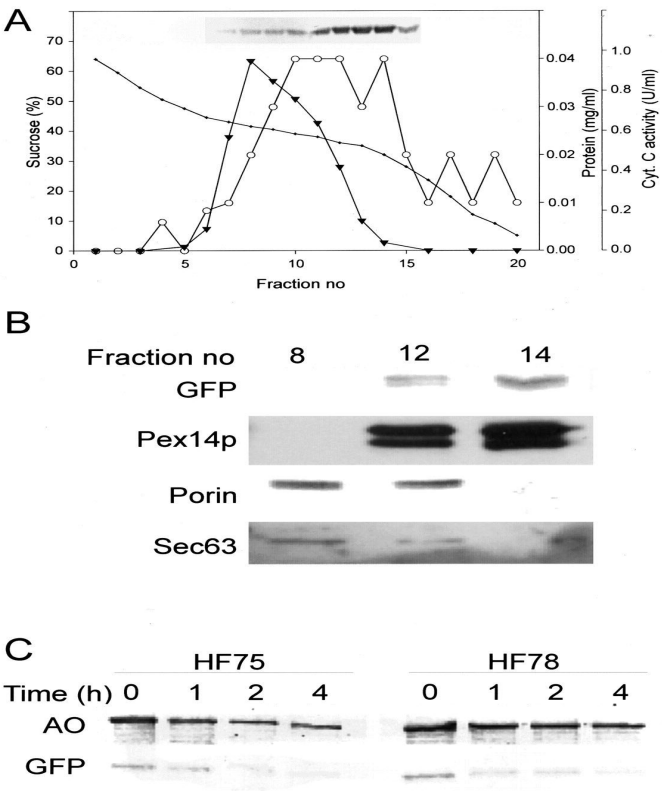

***Fig. 3**. Pex3p$_{[1-50]}$-vesicles harbour peroxisomal characteristics. A, a sucrose density gradient with an adapted sucrose density profile was used to fractionate an organellar pellet fraction prepared from glycerol/methanol-grown HF78 cells. Individual fractions were analysed for sucrose density (+), protein concentration (**O**) and mitochondrial cytochrome c oxidase activity (▾). Western blot analysis was used to determine the location of Pex3p$_{[1-50]}$GFP (top panel). Peak fractions of mitochondria were found in fraction 8, whereas Pex3p$_{[1-50]}$GFP was most abundant in fraction 14. B, Western blot analysis of fractions 8, 12 and 14 for Pex3p$_{[1-50]}$GFP, Pex14p, porin and Sec63p. Pex14p cofractionates with the Pex3p$_{[1-50]}$GFP-vesicles. C, selective degradation of Pex3$_{[1-50]}$-vesicles; strains HF75 (WT::P$_{AOX}$-PEX3$_{[1-50]}$GFP) and HF78 (*pex3*:: P$_{AOX}$-PEX3$_{[1-50]}$GFP) were grown in glycerol/methanol-medium to the late logarithmic phase of growth, shifted to glucose-medium and at selected time points samples were taken. Whole cell extracts, representing equal culture volumes, were analysed for the levels of AO and Pex3$_{[1-50]}$GFP by Western blotting.*

(ghosts) in methanol-induced *H. polymorpha pex* cells (Veenhuis et al., 1996). To this end, HF75 (WT::P$_{AOX}$-*PEX3*$_{[1-50]}$*GFP*) and HF78 cells (*pex3*::P$_{AOX}$-*PEX3*$_{[1-50]}$*GFP*) were grown in glycerol/methanol-containing media to induce peroxisome- and Pex3p$_{[1-50]}$-vesicle formation, respectively. Next, these cells were transferred to fresh glucose-media, thus repressing peroxisome- and vesicle-formation, and the fate of AO and GFP was followed in time. As expected, in HF75 control cells both the level of peroxisomal AO and Pex3p$_{[1-50]}$GFP decreased significantly (Fig. 3C) and after 4 h of induction in glucose medium, few small peroxisomes were observed by fluorescence microscopy and electron microscopy (data not shown). In HF78 cells, however, significant amounts of AO remained detectable at this stage, showing that this cytosolic protein was not actively degraded under these conditions (van der Klei

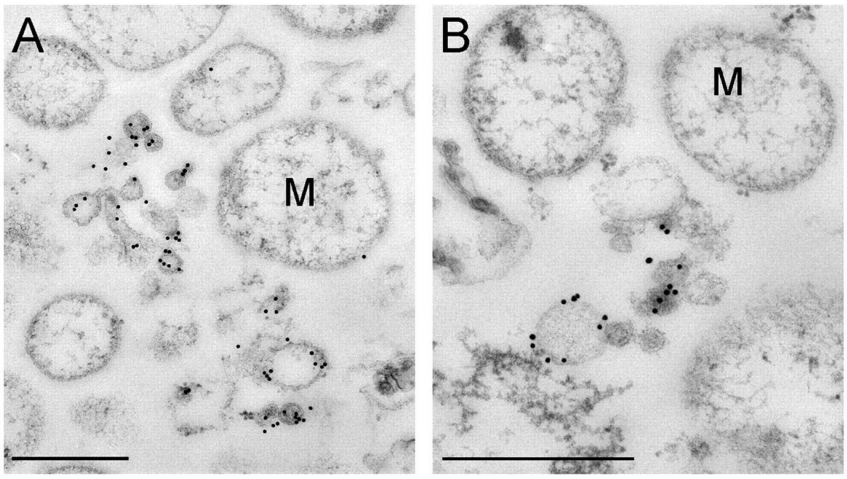

***Fig. 4**. Pex14p is present on Pex3p$_{[1-50]}$-vesicles. Immunolabelling of the organellar fraction loaded onto the sucrose gradient analysed in Fig. 3A, using antibodies against GFP (A) and Pex14p (B). Typical clusters of small vesicles were specifically labelled using these antibodies. Abbreviations: M, mitochondrion. Bar = 0.2 µm.*

et al., 1991). In contrast, the level of Pex3p$_{[1-50]}$GFP decreased with similar kinetics as observed for HF75 cells, suggesting that the Pex3p$_{[1-50]}$GFP-vesicles were actively degraded after a shift of HF78 cells to glucose-medium.

Taken together, these data suggest that the Pex3p$_{[1-50]}$-vesicles are not merely a sink for non-functional proteins, but instead represent membrane vesicles that show peroxisomal properties.

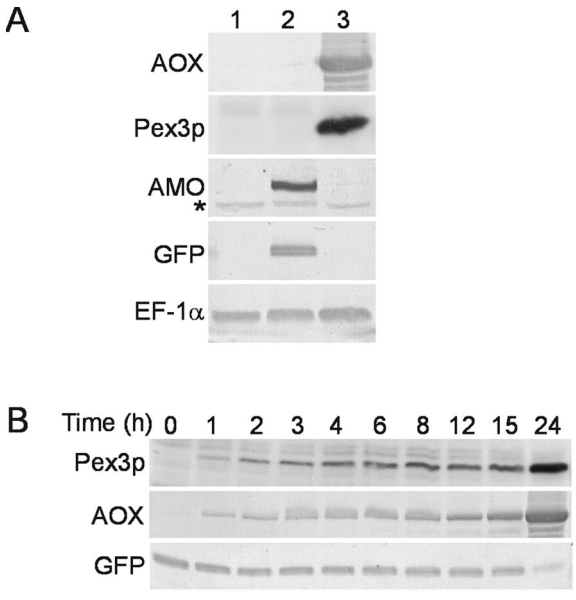

***Fig. 5**. Control of protein synthesis in HF245 cells. A, equal amounts of total protein extracts of HF245 (*pex3*::P$_{AOX}$-PEX3*::P$_{AMO}$-PEX3$_{[1-50]}$GFP) cells, grown in media containing glucose/ammonium sulphate (lane 1), glucose/ethylamine (lane 2) or methanol/ammonium sulphate (lane 3), were analysed by Western blotting for the presence of AOX, Pex3p, AMO, Pex3p$_{[1-50]}$GFP and elongation factor 1α (EF-1α; constitutively synthesised protein used as control). Synthesis of AOX and Pex3p was specifically induced in methanol-containing media, whereas AMO and Pex3p$_{[1-50]}$GFP were detected only in amine-containing media. B, Western blot analysis of total protein extracts of HF245 (*pex3*::P$_{AOX}$-PEX3*::P$_{AMO}$-PEX3$_{[1-50]}$GFP) cells shifted (at t=0) from glucose/ethylamine- to glycerol/methanol/ammonium sulphate medium. One hour after the shift, Pex3p and AOX are detectable, whereas Pex3p$_{[1-50]}$GFP (GFP) remains detectable even after 24 h after the shift.*

Pex3p$_{[1-50]}$-vesicles develop at the nuclear membrane.

Next, we investigated the origin of the vesicles induced upon production of Pex3p$_{[1-50]}$GFP. To this end, we constructed a strain, HF245, in which Pex3p$_{[1-50]}$-vesicle-production and full-length Pex3p synthesis can be separately regulated. This strain is deleted for the endogenous *PEX3* gene. Instead, it contains the *PEX3* gene under control of the methanol-inducible alcohol oxidase promoter (P_{AOX}) together with the gene encoding Pex3p$_{[1-50]}$GFP under control of the amine-inducible amine oxidase promoter (P_{AMO}) (Fig. 5A). HF245 cells were shifted from P_{AMO}-repressing conditions (glucose/ammonium sulphate), to media that induce the P_{AMO} (glucose/ethylamine). Within 1 h after the shift, small fluorescent profiles could be detected in the cells by fluorescence microscopy. After prolonged incubation the brightness of these spots increased and only rarely we observed more than one fluorescent spot per cell (not shown, cf. Fig. 1B). Other fluorescent subcellular structures were not detected by fluorescence microscopy. A detailed electron microscopic analysis of the initial stages of the vesicle proliferation showed that these structures invariably developed in close vicinity of the nuclear membrane (Fig. 6A). Immunocytochemically, Pex3p$_{[1-50]}$GFP was solely detectable at these membranes (Fig. 6B). We never observed the development of a cluster of Pex3p$_{[1-50]}$GFP-containing membranes at another subcellular location. Similar results were obtained when GFP was replaced by β-lactamase as reporter protein. To get further insight in the donor membrane compartment of the Pex3p$_{[1-50]}$-vesicles, we determined whether ER-resident proteins could be detected in, or associated with, the Pex3p$_{[1-50]}$-vesicles by immunocytochemistry. Initial studies were performed using antisera raised against *S. cerevisiae* BiP/Kar2p or Sec63p that cross-react with the *H. polymorpha* orthologues. However, these antisera showed insufficient specificity and/or labelling intensity in the immunocytochemical experiments, also on normal ER. Subsequently, we made use of an artificial marker for the *H. polymorpha* ER lumen. A hybrid protein, consisting of the N-terminal 30 amino acids of *H. polymorpha* BiP (van der Heide et al., 2002), containing its ER sorting signal, and GFP was synthesised in HF290 cells (*pex3*::P_{AMO}-*BiP*$_{[1-30]}$*GFP*::P_{AOX}-*PEX3*$_{[1-50]}$*β-lactamase*) under control of the amine oxidase promoter. Fluorescence microscopic analysis of glucose/ethylamine-grown HF290 cells showed distinct staining of the nuclear envelope together with small patches at the cellular periphery (Fig. 6D). Immunocytochemical analysis showed that in these cells, α-GFP-specific labelling was confined to the nuclear membrane and the lateral ER (Fig. 6C). Next, these cells were shifted to glycerol/methanol/ammonium sulphate-medium, inducing the synthesis of Pex3p$_{[1-}$

$_{50]}$β-lactamase and fully repressing further synthesis of BiP$_{[1-30]}$GFP. Four hours after the shift, these cells were prepared for immunocytochemistry. As expected, synthesis of Pex3p$_{[1-50]}$β-lactamase caused the development of Pex3p$_{[1-50]}$-vesicles at the nuclear membrane. These vesicles contained both the Pex3p$_{[1-50]}$β-lactamase hybrid protein as well as endogenous Pex14p as shown by double-labelling experiments (Fig. 6E). The Pex3p$_{[1-50]}$-vesicles also contain significant α-GFP-specific labelling (Fig.6F, 5-nm gold) suggesting that the artificial marker for the nuclear membrane/ER had incorporated in these vesicles under conditions that its synthesis was fully repressed. Taken together, these data suggest that the Pex3p$_{[1-50]}$-vesicles may be derived from the nuclear membrane/ER.

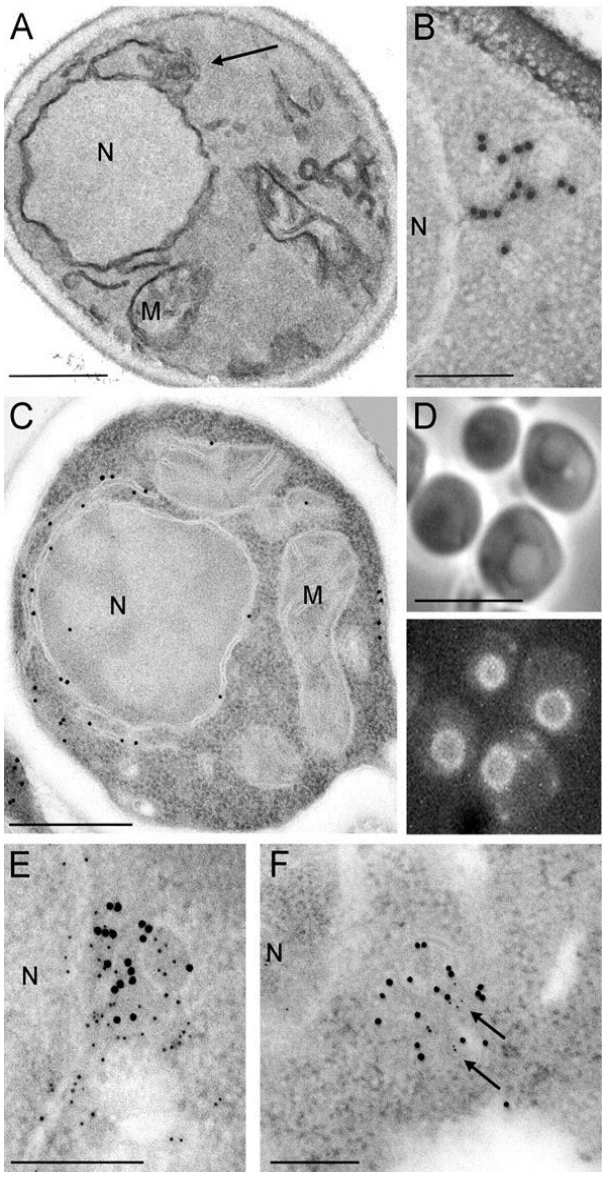

***Fig.6**. Pex3p$_{[1-50]}$-vesicles arise at the nuclear membrane. A,B, electron microscopical analysis of HF245 (*pex3*::P$_{AOX}$*-PEX3*::P$_{AMO}$*-PEX3$_{[1-50]}$*GFP) cells 1 h after a shift from glucose/ammonium sulphate- to glucose/ethylamine-medium. A, Morphology; B, immunolocalisation of Pex3p$_{[1-50]}$GFP using antibodies against GFP. C-F, ER-localised BiP$_{[1-50]}$GFP colocalises with Pex3p$_{[1-50]}$-vesicles. C,D, HF290 (*pex3*::P$_{AMO}$*-BiP$_{[1-30]}$*GFP*::P$_{AOX}$*-PEX3$_{[1-50]}$*β-lactamase) cells were grown in glucose/ethylamine-medium and analysed for the location of BiP$_{[1-30]}$GFP by immunocytochemistry using α-GFP antibodies (C) and fluorescence microscopy (D, top panel, bright field image; bottom panel, fluorescence image). E,F, glucose/ethylamine grown HF290 cells (see C,D) were shifted to glycerol/methanol/ammonium sulphate-medium and after 4 h of growth analysed by immunocytochemistry for the location of Pex3p$_{[1-50]}$ (α-β-lactamase and 5-nm GAR-gold) and Pex14p (α-Pex14p and 15-nm GAR-gold) (E) or BiP$_{[1-30]}$GFP (α-GFP and 5-nm GAR-gold) and Pex14p (α-Pex14p and 15-nm GAR-gold) (F). Abbreviations: M, mitochondrion, N, nucleus. The arrow in A indicates the Pex3$_{[1-50]}$-vesicle cluster. The arrows in F indicate the 5-nm gold particles specific for BiP$_{[1-30]}$GFP.*
Bar = 0.5 µm (A-C), 5 µm (D) and 0.2 µm (E,F).

Pex3p$_{[1-50]}$-vesicles are the target for normal peroxisome development

Since the Pex3p$_{[1-50]}$-vesicles have peroxisomal characteristics, the next question we investigated is whether they can act as precursors for peroxisome biogenesis after re-introduction of Pex3p in these cells. HF245 cells were pre-cultivated in glucose/ethylamine-medium to induce Pex3p$_{[1-50]}$-vesicle formation. Subsequently, these cells were incubated for 30 minutes in glucose-ammonium sulphate-medium to deplete the amine-induced mRNA's. After this incubation, the Pex3p$_{[1-50]}$GFP mRNA level had dropped approximately 1,000-fold as determined by RT-PCR (data not shown). Next, the HF245 cells were transferred to methanol/ammonium sulphate-medium, thus inducing Pex3p synthesis under conditions that fully repress Pex3p$_{[1-50]}$GFP synthesis. Samples were taken at regular time intervals from the HF245 culture and analysed both biochemically and microscopically. Fig. 5B shows that after 1 hour of incubation of these cells in fresh methanol/ammonium sulphate-medium, Pex3p is readily detectable at levels which are slightly higher than those in WT cells grown on glucose/ammonium sulphate (comparison to WT not shown). The high initial levels of Pex3p$_{[1-50]}$GFP at t=0 (glucose/ethylamine) only gradually decreased after the shift (Fig. 5B), suggesting that under these conditions no active degradation occurs of the Pex3p$_{[1-50]}$-vesicles. To analyse the mode and kinetics of peroxisome re-appearance, samples taken at various time points after the shift of cells to methanol were prepared for electron microscopical analyses. As expected, the glucose/ethylamine-grown inoculum cells (t=0) solely harboured GFP-containing vesicles and lacked peroxisomes (not shown, cf. Fig. 1). Four to six hours after the shift, some vesicles within the Pex3p$_{[1-50]}$-membrane clusters had increased in size (Fig. 7A,B). Immunocytochemical staining experiments revealed that AOX protein was present in these enlarged compartments (Fig. 7B, inset). In contrast, Pex3p$_{[1-50]}$GFP (Fig. 7A, inset) and Pex3p (Fig. 7B) were present throughout the whole population of vesicles, not restricted to the membranes of the enlarged vesicles. This suggests that all vesicles apparently initially accumulated Pex3p. However, not all vesicles developed into peroxisomes as indicated by the observation that after prolonged incubation of strain HF245 in methanol-media, relatevely few (3-6) peroxisomes had developed (Fig 7C,D) compared to tne number of vesicles that were originally present. Anti-GFP- (Fig. 7C) and Pex3p- (Fig. 7D) dependent labelling was observed on the membranes of these organelles that were characterised by the presence of AOX protein (Fig. 7D inset). The presence of the GFP marker protein on peroxisomes could also be observed by fluorescence microscopy (Fig. 7C, inset). Since the expression of Pex3p$_{[1-50]}$GFP was fully

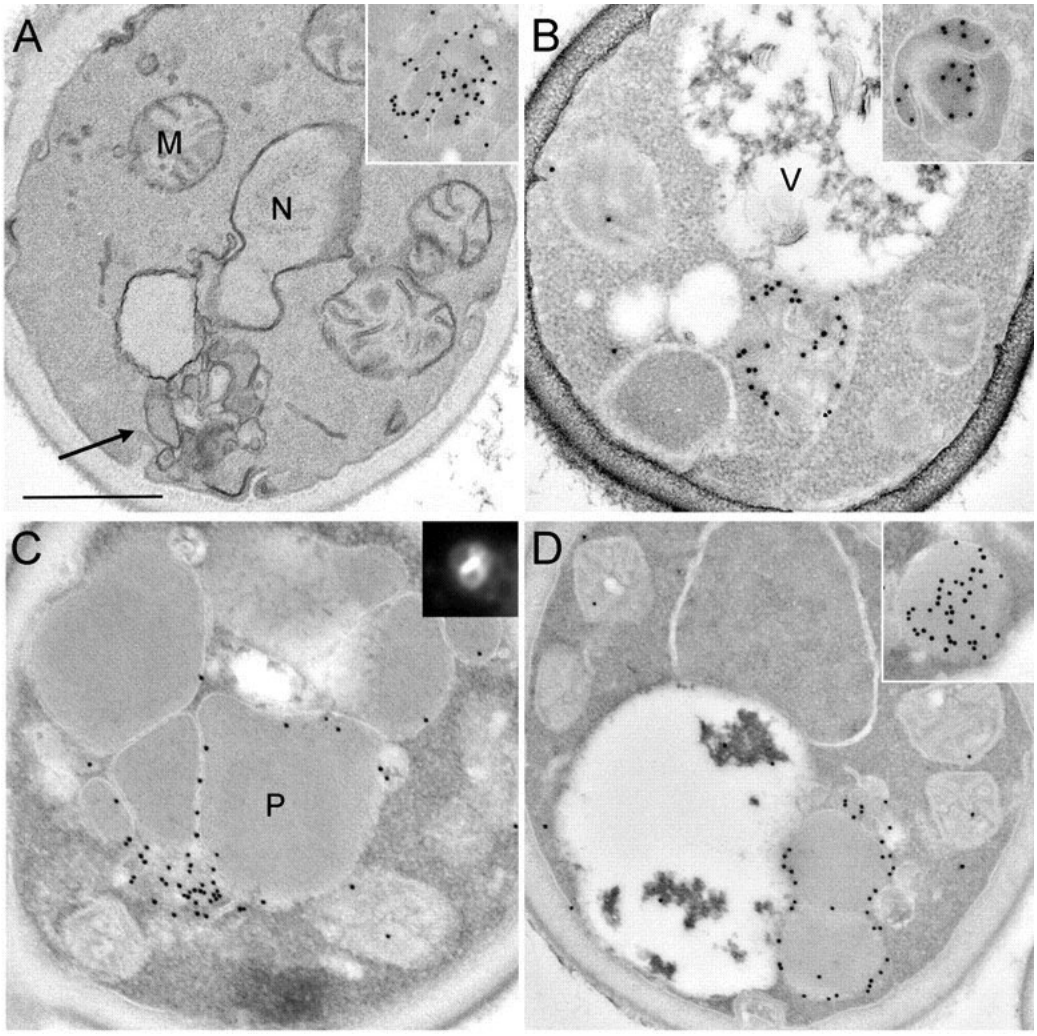

***Fig. 7**. Pex3p$_{[1-50]}$-vesicles are the template for peroxisome biogenesis. Electron microscopic analysis of HF245 (*pex3*::P$_{AOX}$-PEX3*::P$_{AMO}$-PEX3$_{[1-50]}$GFP) cells shifted from glucose/ethylamine-medium to glycerol/methanol/ammonium sulphate-medium. A,B, 6 h after the shift; C,D, 16 h after the shift. A, morphology; B-D and insets, immunolocalisation of Pex3p$_{[1-50]}$GFP (A inset, C); Pex3p (B,D) and AOX (B inset, D inset). Pex3p and AOX are sorted to the Pex3p$_{[1-50]}$GFP-vesicle clusters (B). 16 h after the shift, significant amounts of Pex3p$_{[1-50]}$GFP is found on the peroxisomal membrane (C). The inset in C shows a fluorescent image of HF245 cells 14h after the shift to methanol/ammonium sulphate-medium. Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The arrow in A and E indicates the Pex3p$_{[1-50]}$-vesicle cluster. Bar = 0.5 µm (similar magnifications were used for A-D).*

repressed under these conditions, these data suggest that the newly formed peroxisomes had developed from the Pex3p$_{[1-50]}$GFP-containing vesicles. Subsequently, biochemical experiments were performed on HF245 cells, grown for 22 h after a shift to from glucose/ethylamine- to methanol/ammonium sulphate-medium. Upon gradient centrifugation of cell homogenates minor, but significant amounts of Pex3p$_{[1-50]}$GFP were detected in the peroxisomal peak fraction (at 54% sucrose), where also AOX protein had accumulated (Fig. 8). Taken together,

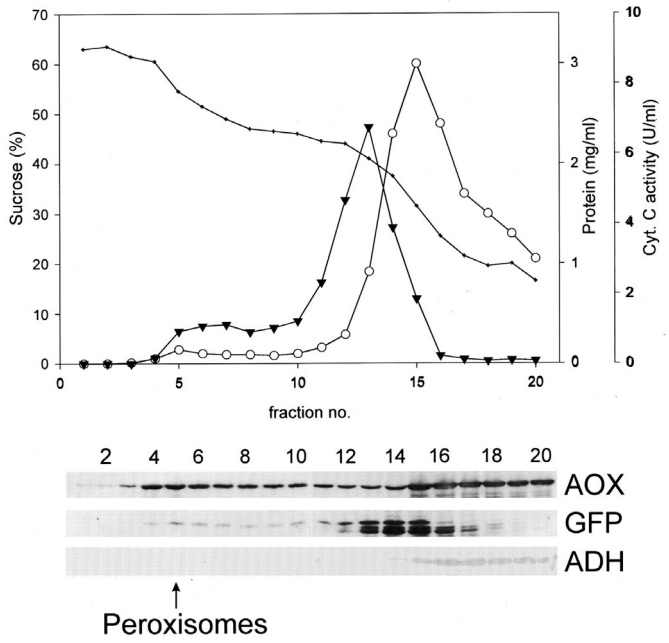

***Fig. 8**. Peroxisomes of shifted HF245 cells contain Pex3p$_{[1-50]}$GFP. Sucrose density gradient centrifugation of a PNS obtained from HF245 (*pex3*::P_{AOX}-PEX3::P_{AMO}-PEX3$_{[1-50]}$GFP) cells pre-grown in glucose/ethylamine-medium, shifted to methanol/ammonium sulphate medium and harvested at 22 h after the shift. Individual fractions were analysed for sucrose density (+), protein concentration (**O**), mitochondrial cytochrome c oxidase activity (▾) and cytosolic ADH protein (Western blot). Western blot analysis on individual fractions shows co-localisation of AOX and Pex3p$_{[1-50]}$GFP at a sucrose density (54%) typical for WT peroxisomes. In addition, significant amounts of Pex3p$_{[1-50]}$GFP was found at sucrose concentrations of approximately 40%, indicating that Pex3$_{[1-50]}$-vesicles are still present in these cells. Also AOX was found at the top of the gradient due to leakage of matrix proteins from damaged peroxisomes during the isolation procedure.*

these data suggest that the Pex3p$_{[1-50]}$-vesicles can act as a template for peroxisome development after subsequent re-introduction of Pex3p.

Complementation of *pex3* cells by full length Pex3p initiates with a single peroxisome.

In control experiments, we analysed peroxisome recovery in *pex3* cells upon complementation by the *PEX3* gene in the absence of Pex3p$_{[1-50]}$-vesicles. Electron microscopy showed that peroxisomes arose within 1 h after the shift of cells to inducing conditions. Invariably, only a single organelle was formed, located in the vicinity of the nuclear membrane (Fig. 9). The organelle was characterised by the presence of Pex3p (Fig. 9B) and AOX (Fig. 9C). However, we never observed any proliferation of vesicles or additional membranes at the initial hours of peroxisome re-introduction comparable to Pex3p$_{[1-50]}$-producing cells. This indicates that the morphological events of peroxisome re-introduction in *pex3* cells significantly differ from those in similar cells that produce Pex3$_{[1-50]}$ protein.

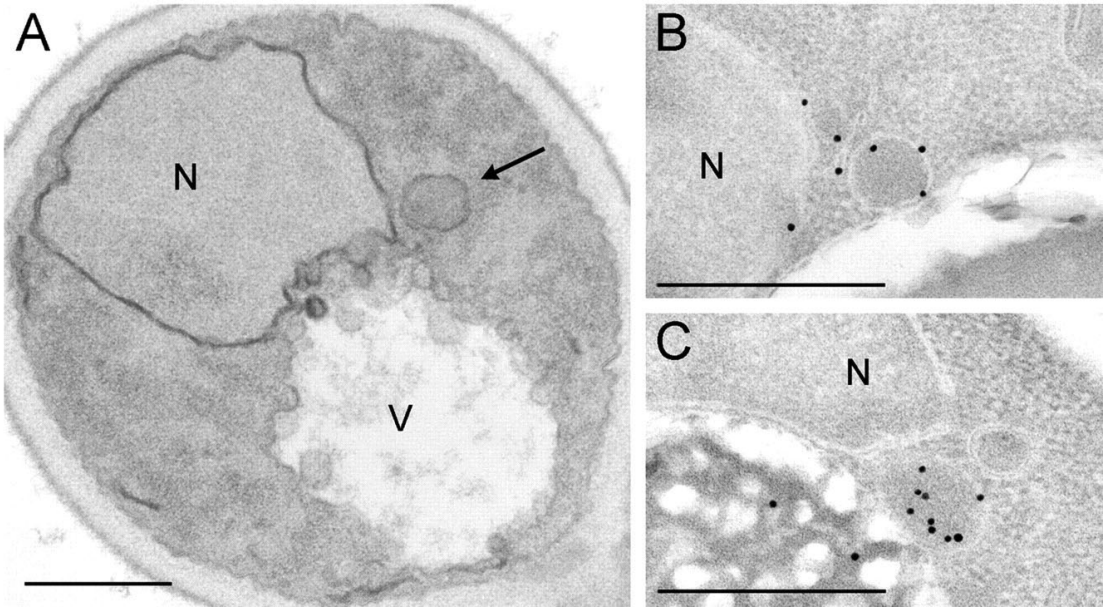

Fig. 9 *Peroxisomes arise at the nuclear membrane upon complementation of* pex3 *cells. A-C electron microscopical analysis of RBG17 (*pex3*::P_{AOX}*-PEX3*) cells 2 h after a shift from glucose/ethylamine-medium to methanol/ammonium sulphate-medium. A. morphology; B,C, immunolocalisation of Pex3p (B) and AOX (C). Abbreviations: N, nucleus, V, vacuole. The arrow in A indicates a newly-formed peroxisome. Bar = 0.5 μm.*

Discussion

Pex3p is a peroxisomal membrane protein essential for peroxisome biogenesis and maintenance of the organellar membrane (Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996; Hettema et al., 2000; South et al., 2000). Previously, we reported that the first 50 amino acids of Pex3p are sufficient to target a reporter protein to the peroxisomal membrane (Baerends et al., 1996; Baerends et al., 2000). Here we show that the same fragment of the protein is capable of inducing vesicle formation in *pex3* cells. These vesicles develop in the vicinity of the nuclear membrane and preferentially served as templates for peroxisome development upon subsequent re-introduction of the full-length Pex3p. These data imply that the Pex3p$_{[1-50]}$-vesicles may be considered as an incomplete peroxisomal compartment that may be 'trapped' in its maturation because of the absence of essential functions in the missing C-terminal part (amino acids 51-457) of Pex3p.

The current view of peroxisome proliferation predicts that new peroxisomes form from pre-existing ones (Lazarow and Fujiki, 1985). However, this model can not explain some recent observations. Specifically, newly formed peroxisomes in peroxisome-deficient cells lacking any detectable peroxisomal (membrane) remnants

(human and yeast *pex3* cells, human *pex16,* human and *S. cerevisiae pex19* cells) require an alternative membrane origin upon introduction of the complementing gene. Also, in *Y. lipolytica* and in plant cells, several peroxisomal membrane proteins have been proposed to travel via the ER or an ER-like compartment before reaching the peroxisome (Titorenko and Rachubinski, 1998; Mullen et al., 1999). Whether this mechanism is generally valid, remains to be elucidated. In *H. polymorpha* an ER-to-peroxisome sorting pathway may exist, exemplified by the finding that a hybrid protein consisting of the 16 N-terminal amino acids of Pex3p fused to a reporter protein (PTS1-less catalase) is targeted to the nuclear membrane (Baerends et al., 1996). The Pex3p$_{[1-50]}$-GFP-induced vesicles described in this study may concentrate at certain subdomains of the apparent donor membrane compartment, the nuclear membrane. Remarkably, upon induction solely the vesicles displayed fluorescence; invariably no nuclear or ER fluorescence was detected. The finding that only vesicles showed fluorescence lends support to the notion that they may not or hardly contain typical ER characteristics. Further evidence that the Pex3p$_{[1-50]}$-vesicles may indeed originate from the endomembrane system comes from the observation that they contained an overproduced ER-lumen protein, BiP$_{[1-30]}$GFP.

The Pex3p$_{[1-50]}$-vesicles contain at least one other peroxin, Pex14p, a protein that is missorted to mitochondria in *pex3* cells (Baerends et al., 2000; South et al., 2000). Because most peroxins in *H. polymorpha* are low abundant and therefore difficult to detect at endogenous levels, we have not been able to show conclusively that other peroxins are also present on these vesicles. The Pex3p$_{[1-50]}$-vesicles have, however, also acquired a typical property of WT *H. polymorpha* peroxisomes in that they are susceptible to selective degradation (Veenhuis et al., 1996).

Interestingly, also in mammalian *pex3* cells a hybrid protein consisting of the N-terminal 40 amino acids of rat Pex3p and GFP is observed in vesicular structures (Ghaedi et al., 2000). These vesicles were however not further characterized, thus it remains to be determined whether also these structures have peroxisomal features.

The key question to be answered was: are the Pex3p$_{[1-50]}$-vesicles a preferred target for peroxisome re-introduction upon synthesis of Pex3p? Using an *H. polymorpha* strain, in which vesicle-formation and peroxisome biogenesis can be separately regulated, we were able to show that the vesicles population accumulated Pex3p, while only few of these incorporated the peroxisomal matrix protein AOX. The possibility that the synthesis of Pex3p itself induces formation of new vesicle-clusters which mix with the pre-existing Pex3p$_{[1-50]}$GFP vesicles is not very likely, because such vesicles were not observed when under identical conditions peroxisomes were

re-introduced in *pex3* cells in the absence of Pex3p$_{[1-50]}$-vesicles (Baerends et al., 1996) (see also Fig. 9).

Our data support the notion that 1) a Pex3p-receptor moiety is likely to exist on the endomembrane system of *pex3* cells and, subsequently, on the Pex3p$_{[1-50]}$-induced vesicles and 2) Pex3p$_{[1-50]}$-vesicles may act as a template for the assembly of peroxisomes. Most likely, incorporation of Pex3p solely does not restore the import of peroxisomal matrix proteins into these vesicles and hence, a further development of these vesicles (e.g. by the uptake of other essential proteins) is required for this. The details of this process, however, remain to be resolved. It can be envisaged that the endogenous levels of such proteins may be too low to complement all vesicles and, in line with this, the transformation of some of the vesicles into peroxisomes may simply occur by chance.

In recent studies Titorenko and co-workers described the function of pre-peroxisomal vesicles in peroxisome biogenesis in WT *Y. lipolytica* cells (Titorenko et al., 2000; Titorenko and Rachubinski, 2001). Five subpopulations (P1-P5) with different biochemical characteristics were discriminated in a high-speed (200,000x g)-pellet fraction of a post-nuclear supernatant after removing the mature peroxisomes during a low-speed (20,000x g) centrifugation step. After a Pex1p- and Pex6p-dependent fusion of P1 and P2, they found that the resultant P3 was a precursor for P4, leading via P5 to mature peroxisomes, implying a prescribed maturation machinery for peroxisomes to become physiologically functional.

Other researchers have questioned the existence of vesicle-mediated, ER-to-peroxisome sorting pathways. Experiments on *S. cerevisiae* Pex15p, suggested that this protein was sorted via the ER, primarily because overproduction of the protein in WT cells caused massive overproliferation of ER membranes (karmellae; (Elgersma et al., 1998). In a recent study however it was shown that endogenous Pex15p remains cytosolic in *pex3* cells whereas overproduced Pex15p again, gave rise to karmellea formation in these cells (Hettema et al., 2000). This led the authors to conclude that the nuclear and ER localisation of the protein, as well as the karmellae formation, may represent an artefact due to Pex15p overproduction.

In human cells, no evidence was obtained for routing of Pex16p via the ER. Like *pex3* cells, human cells lacking functional Pex16p, do not contain peroxisomal ghosts. Re-appearance of peroxisomes in *pex16*-mutant cells upon re-introduction of the *PEX16* gene was not inhibited by BFA and occurred normally at 15^{o}C, conditions that block COPII-mediated protein exit from the ER and COPI-mediated transport from the ER/Golgi intermediate compartment, respectively (South and Gould, 1999).

Essentially similar results have recently been reported for peroxisome rescue in human *pex3* cells upon reintroduction of the complementing gene (South et al., 2000). These authors proposed a two-pathway model of peroxisome biogenesis. These include one pathway confirming to the widely accepted view that peroxisomes arise by the growth and division of pre-existing ones (Lazarow and Fujiki, 1985), and an alternative pathway by which peroxisomes form from a preperoxisomal vesicle. Their analyses, however, gave no clue as to whether such pre-peroxisomal structure exists or whether peroxisomes arise from the endomembrane system.

In WT *H. polymorpha* cells we have at present no direct evidence for a constitutive process invoking a role of ER vesicles in peroxisome biogenesis. However, our present observations suggest that specific vesicles derived from the endomembrane system or nuclear membrane may develop into normal peroxisomes. These data lend support to the view that in WT conditions sorting of Pex3p via the ER might be redundant and that, upon induction by growth compounds, peroxisomes normally develop by growth and fission. However, in cases peroxisomes are lost, e.g. due to chemical-induced damage or failure in inheritance, formation of the organelles might be rescued and initiated at the endomembrane system.

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Chapter 5

Re-assembly of peroxisomes in *Hansenula polymorpha pex3* cells upon re-introduction of Pex3p involves the nuclear envelope

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Abstract

We analyzed the re-assembly of peroxisomes in *Hansenula polymorpha pex3* cells upon reintroduction of Pex3p. Within one hour after the onset of Pex3p production, a single organelle developed per cell, invariably in close proximity of the nuclear envelope. Subsequently, this organelle increased in size, migrated to a position in the vicinity of the cell wall, and multiplied by division. Fractionation experiments on homogenates of *pex3* cells, in which the endomembrane system was tagged with GFP, identified a small amount of GFP in peroxisomes present in the initial stage of peroxisome re-assembly. Taken together, our data suggest a distinct role for the endomembrane system in peroxisome re-assembly in complemented *pex3* cells.

Introduction

Peroxisomes are multi-functional organelles, which play an important role in cellular metabolism (for a review, see (De Duve, 1983; van der Klei and Veenhuis, 1997). Several peroxisome-associated disorders are identified in man, some of which are lethal (e.g. Zellweger syndrome; reviewed in (Wanders, 1999; Gould and Valle, 2000)). In yeasts, peroxisomes are generally involved in the primary metabolism of specific carbon and/or nitrogen sources used for growth (Veenhuis, 1992). Since peroxisomes lack DNA, all peroxisomal proteins, both soluble and membrane-bound, are post-translationally imported into the organelle (Fujiki et al., 1984).

We have isolated peroxisome-deficient (*pex*) mutants of the methylotrophic yeast *Hansenula polymorpha* and cloned seventeen of the corresponding genes. One of these, *PEX3*, encodes a 52 kDa protein essential for peroxisome biogenesis and maintenance (Baerends et al., 1996). The importance of Pex3p in peroxisome biogenesis is underlined by the absence of detectable peroxisomal membrane remnants (ghosts) in a *pex3* deletion strain, a characteristic that is shared only by the human *pex16* and *Saccharomyces cerevisiae pex19* mutant phenotypes (Honsho et al., 1998; Götte et al., 1998). All other *pex* mutants identified thus far contain ghosts.

Although ghosts are missing in the *pex3* mutant, re-introduction and expression of the *PEX3* gene in such mutants resumes peroxisome biogenesis (Baerends et al., 1996; Ghaedi et al., 2000). The origin of the membrane of the newly formed organelles was still enigmatic. The classical model of peroxisome biogenesis involves growth and multiplication of existing organelles by fission, which implies that new peroxisomes develop from pre-existing ones (Lazarow and Fujiki, 1985). However, data that support the possibility of alternative pathways for peroxisome

biogenesis have accumulated recently (for a review, see Titorenko and Rachubinski, 2001).

In this paper we describe that the re-introduction of normal peroxisomes in *H. polymorpha pex3* cells upon expression of the *PEX3* gene proceeds rapidly and involves the nuclear envelope.

Materials and methods

Microorganisms and growth conditions

H. polymorpha strains were grown in batch cultures at 37°C on mineral medium (van Dijken et al., 1976) supplemented with 0.5% carbon source (i.e. glucose or methanol) in the presence of 0.25% ammonium sulphate or ethylamine as nitrogen source. For growth on agar plates, all media were supplemented with 1.5% granulated agar. *Escherichia coli* DH5α (*supE*44 *ΔlacU*169 (ϕ80*lacZΔ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) (Sambrook et al., 1989) was used for recombinant DNA procedures and was grown on LB-medium supplemented with the appropriate antibiotics.

DNA procedures

H. polymorpha was transformed by electroporation (Faber et al., 1994b). Recombinant DNA manipulations were performed essentially as described (Sambrook et al., 1989). Biochemicals were obtained from Roche (Almere, The Netherlands). Site-specific integration at the *AMO*-locus was performed as outlined by Faber *et al*. (Faber et al., 1994a). Southern blotting was performed using the ECL direct nucleic acid labelling and detection system, as described by the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, England). pHIPZ5-BiP$_{[1-30]}$GFP was constructed as follows: The 1.8 kb *Sma*I/*Spe*I fragment of plasmid pFEM76 (Faber et al., 2002) was cloned into the 4.3 kb *Sal*I(Klenow-treated)/*Spe*I fragment of pHIPZ6-Nia.

The *H. polymorpha pex3*::P$_{AOX}$*PEX3*::P$_{AMO}$ *BiP*$_{[1-30]}$*GFP* strain, which can be used to independently express the *PEX3* and *BiP*$_{[1-30]}$*GFP* open reading frames in a *pex3* mutant background, was constructed as follows. *Nar*I-linearized pZ5-BiP$_{[1-30]}$GFP was used to transform *H. polymorpha pex3*::P$_{AOX}$*PEX3* (Baerends et al., 1997). Southern blot analysis of selected zeocin-resistant transformants was performed to verify proper site-specific integration of the expression cassette at the *AMO*-locus in a single copy (data not shown).

Biochemical methods

Protoplasts were prepared and homogenized as described (van der Klei et al., 1998). Cell fractionation experiments were performed as outlined (van der Klei et al., 1998). Cytochrome c oxidase activities were determined as described (Douma et al., 1985). Protein concentrations were determined using the BioRad protein assay kit (BioRad GmbH, Munich, Germany), using bovine serum albumin as standard. SDS-PAGE and Western blotting were carried out as described (Laemmli, 1970; Kyhse-Andersen, 1984). Blots were decorated using the chromogenic (NBT-BCIP) or chemiluminiscent (POD) Western Blotting kit (Roche), using specific polyclonal rabbit antibodies.

Microscopical techniques

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described (Waterham et al., 1994). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific polyclonal antibodies against various peroxisomal proteins or GFP, and gold-conjugated goat-anti-rabbit or goat-anti-mouse antibodies (Waterham et al., 1994). Fluorescence microscopy was performed as described (Baerends et al., 2000).

Results

Reintroduction of Pex3p leads to rapid formation of peroxisomes in pex3 cells

We studied the kinetics of peroxisome re-assembly in *pex3* cells of *H. polymorpha*, upon reintroduction of Pex3p or a Pex3p-GFP fusion by fluorescence and electron microscopical methods. To this end *pex3* strains were constructed that contained a single copy of either the *PEX3* gene, or a *PEX3-GFP* fusion under control of the inducible alcohol oxidase promoter integrated in the genome (*pex3*::P_{AOX}*PEX3*, and *pex3*::P_{AOX}*PEX3-GFP*, respectively) (Baerends et al., 1997). Growth experiments indicated that cells of both transformants had regained the capacity to grow on methanol (data not shown). We analyzed the initial stages of adaptation of the cells from glucose (in which the P_{AOX} is fully repressed) to methanol, which induced the P_{AOX} and thus Pex3p or Pex3p-GFP synthesis. Fluorescence microscopy showed that *pex3*::P_{AOX}*PEX3-GFP* cells grown on glucose displayed no fluorescence, as expected (not shown). However, within one hour of culturing on methanol in each cell a single, strong fluorescent spot appeared (see Fig.1). These spots increased in size with time and subsequently multiplied (not shown) to result in the normal WT

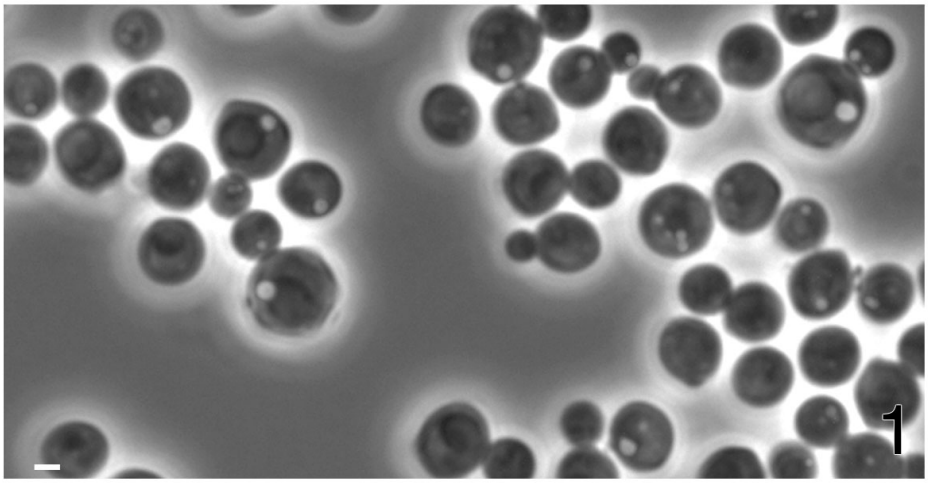

Fig.1*: Fluorescence microscopy of reintroduction of Pex3p-GFP in* Δpex3 *cells. A single fluorescent spot is observed one hour after the onset of expression of* PEX3-GFP *by methanol. Bar represents 1 µm.*

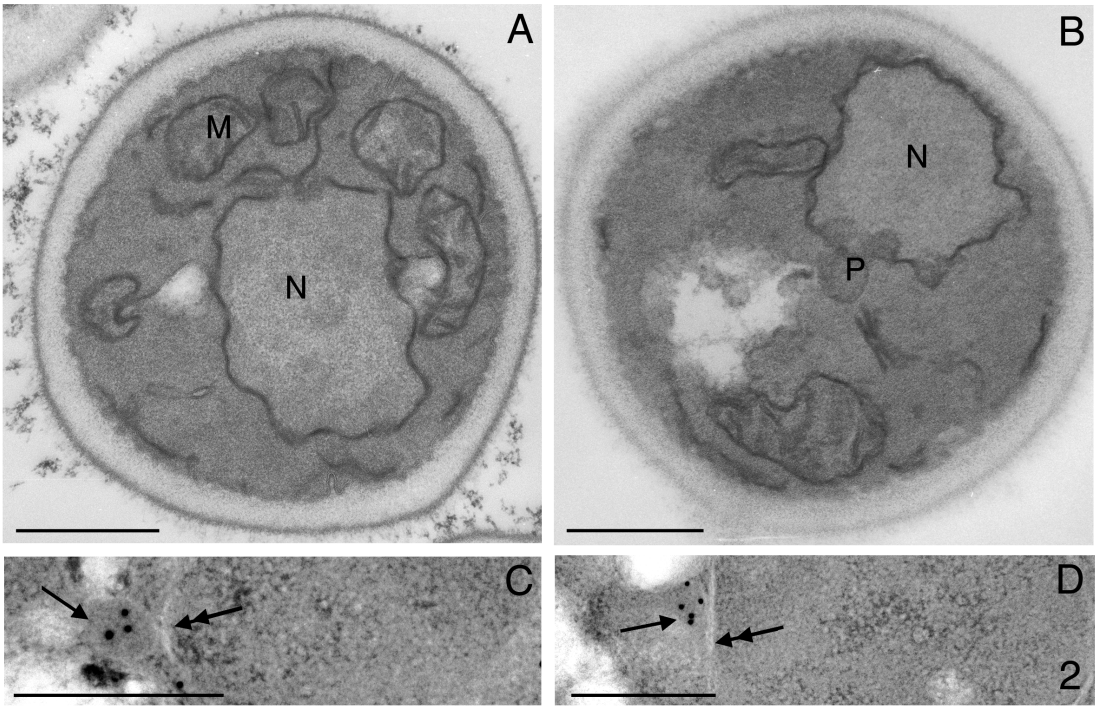

Fig. 2*: Electron microscopical analysis of reintroduction of Pex3p in* Δpex3 *cells.* ***A:*** *Morphology of strain* pex3::P_{AOX}PEX3 *grown on glucose. Peroxisomes are lacking completely.* ***B****:* pex3::P_{AOX}PEX3 *cells 1 hour after shift to methanol-containing medium. A single, small peroxisome is present in the vicinity of the nuclear membrane.* ***C, D****: Immunocytochemical analysis of* pex3::P_{AOX}PEX3 *cells after 1 hour of growth in methanol-containing medium using α-AOX antibodies. The matrix of the newly formed peroxisomes is specifically labeled. Arrow indicates peroxisome, double-headed arrow indicates nuclear membrane. M, mitochondrium, N, nucleus, P, peroxisome. Bars represent 0.5 µm.*

phenotype of cells containing several spots (Baerends et al., 2000). These

observations were confirmed by electron microscopical data, shown in Figure 2, which revealed that in all cells a small peroxisome could be observed within 30 minutes after the shift to methanol. Remarkably, the cells generally contained only one peroxisome that increased in size during further cultivation. This organelle was invariably observed in close proximity of the nuclear envelope. Immunocytochemistry showed that these structures contain the peroxisomal membrane protein Pex3p, as well as the matrix protein AO, and therefore indeed represent developing peroxisomes (Fig. 2). These analyses also demonstrated that peroxisome re-introduction in both *pex3*::P_{AOX}*PEX3*, and *pex3*::P_{AOX}*PEX3-GFP* cells proceeded identically.

Reintroduction of Pex3p in pex3 cells, that artificially produce ER-resident GFP, leads to reassembly of peroxisomes that contain GFP

In order to enable microscopical and biochemical distinction of ER-type membranes from other subcellular compartments, we fused the N-terminal 30 amino acids from the *S. cerevisiae* ER-located Hsp70 protein BiP to GFP. We showed before that this portion of BiP is sufficient to sort reporter proteins to the ER of *H. polymorpha* (van der Heide et al., 2002). The constructed strain, *pex3*::P_{AOX}*PEX3*::P_{AMO} *BiP*$_{[1-30]}$*GFP*, was grown on glucose/ethylamine-containing media. Under these conditions the alcohol oxidase promoter (P_{AOX}) is fully repressed (by glucose), wereas the amine oxidase promoter (P_{AMO}) is induced by the amine nitrogen source. Fluorescence microscopy, using cells from the mid-exponential growth phase on glucose/ethylamine, showed distinct fluorescence of the nuclear envelope and the peripheral ER (see Figure 3). Subsequently, this strain was transferred to conditions

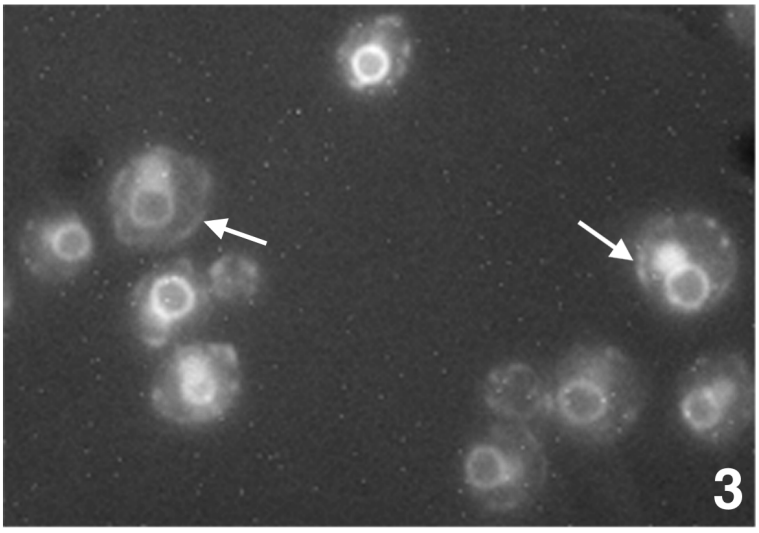

Fig. 3*: Fluorescence microscopy of* pex3*::*P_{AOX}PEX3*::*P_{AMO} BiP$_{[1-30]}$GFP*. Fluorescence can be observed in the nuclear membrane, as well as in peripheral ER (indicated by arrows). Some cells also show fluorescence inside the vacuole.*

that induce peroxisome biogenesis. To this purpose *H. polymorpha pex3*::P_{AOX}*PEX3*::P_{AMO} *BiP*$_{[1-30]}$*GFP* cells were grown to the mid-exponential logarithmic growth phase on glucose/ethylamine until OD_{663} = 1.5, harvested by centrifugation and subsequently resuspended for 30 min in fresh glucose/ammoniumsulphate media at 37 °C, conditions that were previously established to fully deplete P_{AMO}-induced mRNAs (Waterham et al., 1993). Next, the cells were transferred to fresh methanol/ammonium sulphate containing media to induce the WT *PEX3* gene, thereby reintroducing functional Pex3p under conditions that fully repress BiP$_{[1-30]}$GFP synthesis (by ammonium ions).

Western blot analysis of crude extracts prepared from these cells shows that during adaptation of the cells to the new methanol environment, both AO and Pex3p are induced, as expected (Fig. 4). Two hours after the shift of cells to methanol, distinct

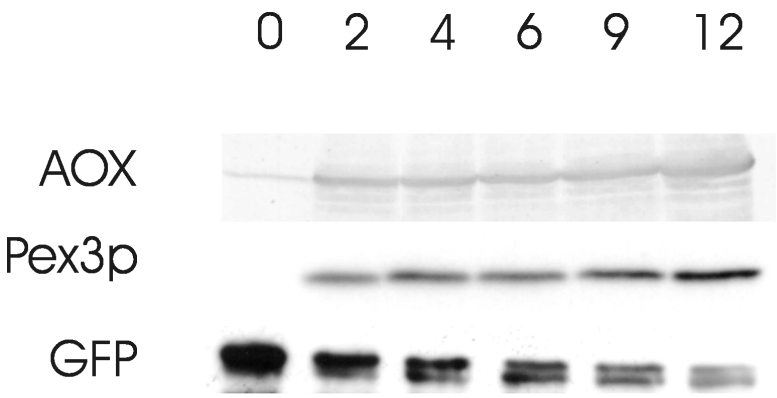

Fig. 4*. Protein levels in* Δpex3*::*P_{AOX}PEX3*::*P_{AMO} BiP$_{[1-30]}$GFP *cells during adaptation on methanol/ammoniumsulfate medium. Crude extracts of cells harvested at indicated timepoints (in hours) were subjected to SDS-PAGE and Western blotting. AOX: alcohol oxidase; GFP: Bip$_{[1-30]}$GFP.*

levels of both proteins can be readily detected. The level of GFP, which is present at high levels at the time of the shift (designated T_0), is gradually decreasing during prolonged growth of cells on methanol and decreased to approximately 10% of the initial level after 12 h of incubation.

Immunocytochemistry revealed that 8 hours after the shift of cells to methanol, peroxisomes were present which contained Pex3p and the peroxisomal matrix protein AO (see Fig. 2). Significant labeling of peroxisomes using GFP-specific antibodies was not observed in immunocytochemical experiments, indicating that GFP levels were below the detection limit (not shown). Also, fluorescence microscopy could not resolve the initially developing peroxisomes in the strong fluorescence background of the nuclear envelope/ER-borne GFP.

Subcellular fractionation of lysates of *pex3*::P_{AOX}*PEX3*::P_{AMO} *BiP*$_{[1-30]}$*GFP* cells prior to Pex3p reintroduction (T_0) shows that Pex3p and AO protein are absent in all gradient fractions, as expected (see Figure 5). BiP$_{[1-30]}$GFP largely sedimented to a protein peak in fraction 14 (42 % sucrose), close to the ER membrane marker Sec63p. The

cytosolic marker protein alcohol dehydrogenase (ADH) is found at the top of the gradient. The activity of cytochrome c oxidase, the mitochondrial marker enzyme, is mainly present in fractions 11-16 (46-40 % sucrose). Eight hours after incubation of cells in methanol/ammonium sulphate containing media (T_8), Pex3p and AO are readily detectable in the sucrose density gradient. Both proteins are detected throughout a large part of the gradient (fractions 4-20 for AO, 5-15 for Pex3p) in conjunction with a minor peak of both proteins at fraction 5 (55% sucrose). This corresponds to the expected position of WT *H. polymorpa* peroxisomes (van der Klei et al., 1998). This observation suggests that minor portions of the newly synthesised peroxisomal proteins AO and Pex3p reside in structures that display characteristics of normal WT peroxisomes. However, significant quantities of both proteins are found in fractions with lower density, possibly indicating the presence of these proteins in structures of lower density or leakage (in case of AO). The bulk of $BiP_{[1-30]}$GFP largely co-localizes with Sec63p, as is the case at T_0. However, a minor but significant portion of GFP can be detected in higher density fractions, co-localizing with AO and Pex3p. This can not be due to the presence of contaminating cell membrane vesicles or (fragmented) protoplasts that carry cytoplasmic components as is indicated by the distribution of the cytosolic marker protein ADH. Therefore, these data suggest that a minor amount of $BiP_{[1-30]}$GFP is now present in peroxisomes. The controls, ADH and cytochrome c oxidase, sediment in patterns that are largely super imposable on those found at T_0.

The results of the fluorescence and electron microscopical analyses described above already indicate that only a very small fraction of $BiP_{[1-30]}$GFP can be detected in the newly formed peroxisomes. Taken together, our data lend support to the notion that the nuclear envelope can act as template for the re-introduction of peroxisomes in *pex3*::P_{AOX}*PEX3*::P_{AMO} *BiP*$_{[1-30]}$*GFP* cells.

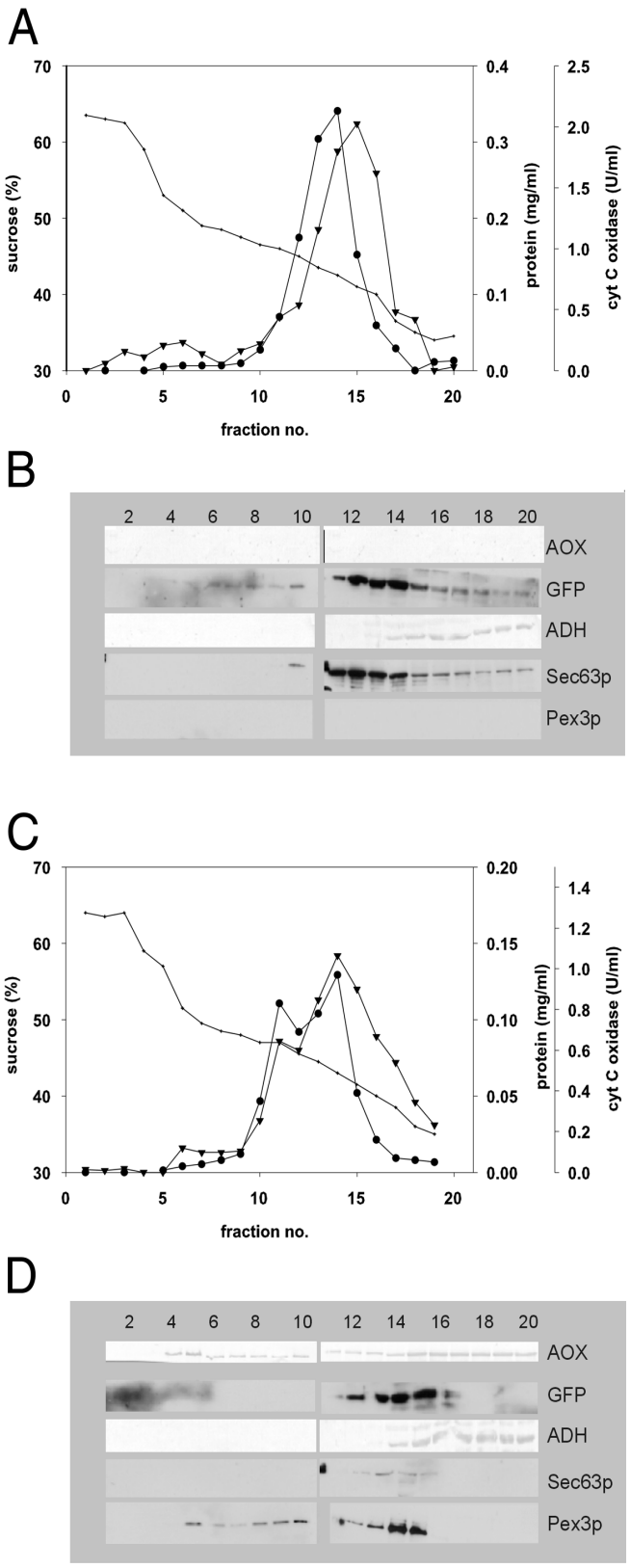

Fig. 5*. Biochemical analysis of reintroduction of Pex3p in* Δpex3 *cells.*

A, B*: Sucrose density gradients of* H. polymorpha Δpex3*::*P_{AOX}PEX3*::*P_{AMO} $BiP_{[1-30]}$GFP *cells grown on glucose/ethylamine.* ***C, D****: Sucrose density gradients of* H. polymorpha Δpex3*::*P_{AOX}PEX3*::*P_{AMO} $BiP_{[1-30]}$GFP *pregrown on glucose/ethylamine, eight hours post-shift to methanol/ammonium.* ***A, C****: +: sucrose percentage; ▼: protein mg* ml^{-1}*; ●: cytochrome C oxidase U* ml^{-1}*.* ***B, D****: Western blots are shown for: AOX: alcohol oxidase; GFP:* $BiP_{[1-30]}$*GFP; ADH: alcohol dehydrogenase; Sec63p; Pex3p as indicated. For detection of Sec63p and ADH, antibodies raised against the* Saccharomyces cerevisiae *homologues, which cross-react with the corresponding* H. polymorpha *proteins, were used.*

Discussion

In this paper we provide evidence for a role of the endomembrane system in the rescue of peroxisomes in *H. polymorpha pex3* cells. *pex3* cells lack morphologically detectable peroxisomal membrane remnants ("ghosts") and thus, hypothetically, a peroxisomal membrane template for peroxisome re-assembly. However, re-introduction of WT Pex3p in the mutant led to the rapid reappearance of a small peroxisome per cell that was invariably localised in close proximity to the nuclear envelope. GFP, accumulated in the ER lumen, including the nuclear envelope, of *pex3* cells appeared to be present in the initial peroxisomes in the complemented cells, suggesting that these membranes served as template for the formation of the organelles.

Previous work in our laboratory has already suggested a putative role of the endomembrane system in one specific case of peroxisome biogenesis. We showed that synthesis of the first 50 amino acids of Pex3p ($Pex3p_{[1-50]}$) resulted in the formation of vesicles that arose from the nuclear envelope (Faber et al., 2002, chapter 4). These vesicles had the potential to develop into normal peroxisomes upon reintroduction of full-length Pex3p. This implies that mature Pex3p - eventually in conjunction with other peroxisomal membrane proteins (PMPs)- can accumulate all components necessary to develop the vesicles into normal peroxisomes and thus, provide indirect evidence that the nuclear envelope can generate the template for peroxisome re-introduction. Our present data link to and extend these findings to more direct line of evidence that the nuclear envelope indeed can serve as template to allow peroxisome rescue in *H. polymorpha pex3* cells.

In *Yarrowia lipolytica* several observations were made that point to an ER - peroxisome assembly relationship. In this organism N-linked core glycosylation of the peroxins Pex2p and Pex16p was observed. This finding suggests that these peroxins have been in contact with the ER lumen during some stage of their presence in the cell (Titorenko and Rachubinski, 1998). Further evidence for a role of the ER in peroxisome biogenesis in this organism came from the observation that the *Y. lipolytica* mutants *sec238* and *srp54*, which are specifically affected in the general secretion route via the ER, are also disturbed in peroxisome biogenesis. Moreover, they accumulate Pex2p and Pex16p in the ER (Titorenko and Rachubinski, 1998). In the same paper, Titorenko *et al*. provide evidence for a multi-step process for peroxisome biogenesis, involving the development of five peroxisomal sub-forms with different characteristics that develop into mature peroxisomes. However, other studies failed to provide evidence for a role for the ER in peroxisome biogenesis in

yeast and human cells (South et al., 2000; South et al., 2001; Voorn-Brouwer et al., 2001).

Rescue of peroxisomes in *pex* mutant cells that lack ghosts has been observed in several organisms. Previous studies on *S. cerevisiae*, *H. polymorpha*, *Pichia pastoris*, *Homo sapiens*, and *Rattus norvegicus pex3* (Höhfeld et al., 1991; Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996; Shimozawa et al., 2000; Muntau et al., 2000; Ghaedi et al., 2000), *Y. lipolytica* and *H. sapiens pex16* (Eitzen et al., 1997; Honsho et al., 1998), and *S. cerevisiae* and *H. sapiens pex19* (Hettema et al., 2000; Matsuzono et al., 1999) revealed that peroxisome biogenesis was restored in these mutants upon reintroduction of the corresponding genes. All three *pex* phenotypes are characterised by the absence of detectable ghosts. The absence of peroxisomal membranes in cells lacking Pex19p has been questioned by the results of Snyder *et al*. in *P. pastoris* and Lambkin and Rachubinski (2001) in *Y. lipolytica*. In *P. pastoris pex19* cells small, Pex3p-containing structures in have been identified that appear to be different from the ghosts, observed in other *pex* mutants (Snyder et al., 1999) whereas in *Y. lipolytica* structures were observed that resemble normal peroxisomes (Lambkin and Rachubinski, 2001). The origin of the newly synthesised peroxisomes is not revealed in detail in any of these studies. In their careful study on the rescue of peroxisomes in a cell line from a Zellweger syndrome patient (PBD061), defective in *PEX16,* upon introduction of the *PEX16* expression vector, Gould and co-workers observed the first new peroxisomal structures in a time span of three hours. On the basis of their data these workers proposed a model for peroxisome rescue in complemented PBD061 cells. This model predicts that Pex16p creates nascent peroxisomes from a yet unidentified structure, termed pre-peroxisome. The nascent peroxisomes subsequently can develop into normal peroxisomes by the import of other PMPs, also including the proliferation factor Pex11p. This is an attractive hypothesis that may also explain how the vesicles that are induced by the synthesis of the first 50 amino acids of Pex3p ($Pex3p_{[1-50]}$) in *H. polymorpha pex3* cells can develop into normal peroxisomes upon synthesis of full-length Pex3p (Faber et al., 2002). Given the fact that $Pex3p_{[1-50]}$ can generate such vesicles, we speculate that the formation of pre-peroxisomes that are predicted in the model of Gould et al., in fact is dependent on Pex3p function. In this view the data of Gould on peroxisome rescue in PBD061 are fully in line with our results in *H. polymorpha pex3* cells. Upon Pex3p synthesis, pre-peroxisomal structures are formed that by the incorporation of other PMP's can develop into normal peroxisomes. However, the biochemical properties of the putative pre-peroxisomes structures are still an enigma. Also, the order of events, e.g. an eventual order of

successive incorporation of PMPs in the pre-peroxisomal structure, if any, is fully unknown. Since initially only a single peroxisome is formed per cell, it is difficult to envisage that peroxisome re-assembly in *H. polymorpha pex3* cells follows a similar pathway as described for the multi-step peroxisome development in *Y. lipolytica* (Titorenko et al., 2000). Also, we have to take into account that the data on *Y. lipolytica* cannot be extrapolated to *H. polymorpha* because the principles of peroxisome biogenesis intrinsically differ between the two organisms. Nevertheless, comparative studies are required to solve this point.

The putative Pex3p-dependent formation of pre-peroxisomes may also explain why we failed to demonstrate a clear-cut GFP fluorescence in the newly formed peroxisomes in *pex3*::P_{AOX}*PEX3*::P_{AMO}*BiP*$_{[1\text{-}30]}$*GFP* cells since it can readily be envisaged that initially formed organelles are very small. Probably, these structures originate at specialised regions of the nuclear envelope (Faber et al., 2002), which may add to an explanation as to why Gould et al. did not observe any biochemical relation between ER functions and peroxisome biogenesis (South et al., 2001).

The bulk-flow hypothesis for soluble ER protein (Wieland et al., 1987) predicts that the ER/nuclear envelope lumen and the vesicles (initial or pre-peroxisomes) derived from it, contain equal concentrations of GFP. After re-introduction of Pex3p, these initial structures rapidly increase in size, this way diluting the original low amount of GFP throughout the expanding volume of peroxisomal matrix, still allowing GFP demonstration by biochemical but not by fluorescent means. Confocal Laser Scanning Microscopy, a technique that allows analysis of stacks of subsequent sections of biological samples containing fluorescent markers, may represent a promising tool to visualize the early events in future studies on the formation of the GFP-containing pre-peroxisomes.

It is relevant to mention here that we speculate that the above mechanism of peroxisome rescue is not a common mechanism in normally induced WT cells. In such cells peroxisome proliferation proceeds via fission of existing organelles. Most likely the rescue mechanism becomes operative in cells that have lost the organelle, for instance due to a failure in inheritance.

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Chapter 6

Summary

Life on earth can be divided into three kingdoms: The Archeae, the eubacteria, and the eukarya. The kingdom of eukarya accommodates all plants, animals, and fungi. The hallmark of eukarya is the presence of a nucleus and other compartments, named organelles, in the eukaryotic cell. Archeae and bacteria lack organelles. Compartmentalization of a cell conceivably enables an organism to perform a great variety of cellular activities with higher efficiency. Metabolic processes that would not proceed in each other's presence can occur simultaneously in different organelles. Another beneficial effect of compartmentalization is surface expansion of internal membranes that perform important functions in many vital processes. Finally, metabolic pathways that yield toxic metabolites can be shielded away from the rest of the cell.

Microbodies are the most recently discovered class of organelles known to date (1954). The initial description of microbodies was exclusively based on their simple morphology. Subsequent biochemical analysis showed that several distinct classes of microbodies existed. Nowadays, microbodies are subdivided into peroxisomes, glycosomes, hydrogenosomes, and glyoxysomes. This study focuses on the peroxisome. A great diversity of metabolic activities takes place inside this organelle. Size, shape, and number of peroxisomes are highly dependent on cell type and physiological conditions. Likewise, these factors greatly impact the metabolic processes that take place in peroxisomes. The presence of peroxisomes is closely fine-tuned with the ever-changing metabolic needs of the cell. This makes the peroxisome a very versatile organelle. The significance of this cell compartment is probably most vividly illustrated by the very grave, sometimes even lethal diseases in man that are associated with disfunctioning of peroxisomes, the **PBD**s (**P**eroxisome **B**iogenesis **D**isorders).

Yeasts are very suitable organisms for studying peroxisome biogenesis. Mutants that show aberrant peroxisome biogenesis (***pex*** mutants) are viable under certain conditions. This enables identification of the affected gene and its protein product. 25 proteins that are directly involved in peroxisome biogenesis, the so-called peroxins, have been discovered thus far. Yeasts provide an excellent model for studying the causes of PBDs. Based on results obtained in yeasts, the molecular causes of 11 out of 12 PBDs have been determined.

For many years the "growth and fission model" has been the central model to explain peroxisome proliferation. In this model, new peroxisomes are exclusively formed from existing ones by a process of growth and fission. When cells divide, each peroxisome in the daughter cell is derived from those present in the mother cell through fission. The growth and fission model excludes the possibility of *de novo* peroxisome

biogenesis (biogenesis “out of the blue”). However, recent research data raise the question whether alternative models might apply. The research described in this thesis was aimed at probing the possibility of peroxisome biogenesis by means other than “growth and fission”. In these studies the methylotrophic (“growing on methanol”) yeast *Hansenula polymorpha* was used. The presence of functional peroxisomes is essential for methylotrophic growth. Hence, *H. polymorpha pex* mutants are unable to grow on methanol media.

Chapter 1 of this thesis reviews our current knowledge of peroxisome biogenesis and function. An overview is given of data indicating peroxisome biogenesis by means other than the growth and fission model.

Chapter 2 describes the isolation of the *H. polymorpha PUR7* gene. This gene was found in a search for the part of the full complement of DNA (genome) of *H. polymorpha* able to restore the defect of the *ade11.1* mutant. This mutant cannot synthesize purine (a DNA-building block) and therefore is unable to grow on media lacking adenine (a purine). The piece of DNA that could restore growth of *ade11.1* in the absence of adenine was found to contain the gene encoding the enzyme that performs the seventh step in purine biosynthesis (*PUR7*). By cloning the *PUR7* gene into a plasmid (a circular DNA that is maintained in the cell independent of the genome) an important genetic tool for *H. polymorpha* was constructed. The resulting plasmid, pHIPA4, was shown to be suited efficiently overproducing proteins in the *ade11.1* mutant. This was tested using two peroxisomal proteins, amine oxidase and catalase. The possibilities for genetic modification of *H. polymorpha* are expanded by using pHIPA4 in the *ade11.1* genetic background. This enhances the applicability of this organism in both fundamental and applied studies.

Some years ago, the Pex3 protein (Pex3p) was identified as a peroxin, localized at the peroxisomal membrane. The *PEX3* gene complemented the *pex3* mutant. The most striking characteristic of the *pex3* mutant is the complete lack of peroxisomal remnants (“ghosts”). A majority of known *pex* mutants does contain ghosts, which indicates an ability to perform normal assembly of the peroxisomal membrane. The lack of ghosts in the *pex3* mutant points towards an important role for Pex3p in peroxisome membrane assembly. Therefore, we chose to study the function of Pex3p in biogenesis of peroxisomes. Chapter 3 describes our research on the localization of Pex3p. We used four variants of Pex3p, harboring a specific, recognizable amino acid sequence built in at different locations in the protein. This sequence, the so-called myc-tag (EQKLISEEDL), was exploited to detect the tagged Pex3p variants. First, the effects of the introduced tags on the function of Pex3p were

tested by checking the ability of the proteins to restore methanol growth of the *pex3* mutant. All Pex3p-myc constructs proved to function as Pex3p and thus enable growth on methanol. The orientation of Pex3p in the peroxisomal membrane was determined by several independent methods. By addition of a protease (a protein capable of digesting other proteins) to intact peroxisomes it was shown that all myc-tags were sensitive for the protease. In another experiment the ability of highly specific anti-myc antibodies (proteins that bind to myc-tags with high affinity) to bind the tags was established. In all four Pex3p-myc variants binding of the anti-myc antibodies occurred, proving that the tags are exposed on the outside of the peroxisomes. Finally, the nature of Pex3p binding to the peroxisome membrane was determined by an extraction experiment. This showed that the protein is firmly attached to the outside of the peroxisome, without actually spanning the membrane. This result is very different from all previously published data. Most likely, Pex3p binds to another protein that is present in the peroxisomal membrane. At this point the identity of this interacting partner is unknown. Further evidence for the localization of Pex3p was obtained by freeze-etch electron microscopy. This method showed that overproduction of Pex3p results in a picture that differs dramatically from the images that can be made when proteins with multiple membrane spanning regions were overproduced. Fluorescence microscopical analysis of cells producing a fusion of Pex3p and GFP (a fluorescent marker protein) yielded a surprising result. The fluorescence was found in very distinct patches on the peroxisome membrane. This concentration in “hot spots” seems to indicate that Pex3p can be found only in clearly defined areas in the organellar membrane.

What events occur when part of Pex3p is produced in *pex3* cells? To our surprise a mutant containing 50 amino acids of the beginning (N terminus) of Pex3p ($Pex3p_{[1-50]}$), harbored great numbers of vesicles. Chapter 4 describes the origin and nature of the vesicles in detail. The vesicles appeared within 30 minutes after the onset of $Pex3p_{[1-50]}$ production, and invariably were found close to the nuclear membrane. Biochemically as well as electron microscopically Pex14p, a peroxisomal membrane protein, was found in the vesicles. Also alcohol oxidase, a peroxisomal matrix protein, was present in the vesicles. This proves that the vesicles have peroxisomal properties. The information needed to transform the vesicles into functional peroxisomes is apparantly present in the part of Pex3p that is lacking in $Pex3p_{[1-50]}$ (i.e. amino acids 51-457). When the full-length Pex3p was produced in vesicle-containing cells, new peroxisomes were formed amidst the vesicles. Further analysis showed that the peroxisomes actually arose from a number of vesicles. Establishing that GFP, used as a marker protein for the vesicles, could also be found inside the

novel peroxisomes proved this point. These results show that structures derived from the nuclear membrane can be used as templates for new peroxisomes under the given circumstances. This is an example of non-“growth and fission” peroxisome biogenesis. The vesicles can be considered as intermediates in the process of peroxisome biogenesis from the nuclear membrane.
Chapter 5 is concerned with the situation arising after full-length Pex3p is reintroduced into mutant *pex3* cells. Under these circumstances a single organelle is formed in close proximity of the nuclear membrane. Using a protein that accumulates inside the ER, (and hence in the nuclear membrane) BiP-GFP, it is shown that the peroxisome most likely generates from a part of the ER, because GFP can be found in the new organelles. The combined data from chapter 4 and 5 show that peroxisomes certainly can derive from the ER, instead of exclusively budding of from pre-existing peroxisomes.

Recent data from several researchers indicates the possibility of peroxisome biogenesis in the absence of pre-existing peroxisomes. The results outlined in this thesis also show the presence of a biogenesis route outside the classic growth and fission model. The exact role of this route in healthy, “wild type”, *H. polymorpha* cells remains unclear. Possibly it never occurs in wild type cells. Instead, it might be a rescue pathway when peroxisomes are lost from the cell, e.g. during cell division. Further research is required to answer the many remaining questions, for instance with respect to the identity of the nuclear membrane receptor for Pex3p.

Chapter 7

Samenvatting

Alle vormen van leven op aarde kunnen worden onderverdeeld in drie rijken: Archeae, eubacteria en eukarya. Deze laatste groep herbergt onder andere de planten, dieren en schimmels. Het kenmerk van eukarya is het bezit van een celkern en andere intracellulaire, door membranen afgesloten compartimenten, de zogenaamde organellen. Archeae en bacteriën bezitten deze niet. Men veronderstelt dat de aanwezigheid van specifieke, in functie verschillende compartimenten in een cel het mogelijk maakt om de grote verscheidenheid aan cellulaire activiteiten zo efficiënt mogelijk te laten verlopen. Zo is het bijvoorbeeld mogelijk om metabole processen, die in elkaars aanwezigheid niet of zeer inefficiënt zouden verlopen, toch tegelijkertijd te laten plaatsvinden. Een ander voordeel van het bezit van organellen is vergroting van het oppervlak van interne membranen, die een belangrijke rol spelen in veel levensprocessen. Verder is het mogelijk om metabole processen die toxische eind of tussenproducten opleveren, fysiek te scheiden van de rest van de cel.

Van alle thans bekende organellen is de microbody (microbody = klein lichaampje) het meest recent ontdekt (1954). Microbodies zijn in eerste instantie uitsluitend omschreven op basis van hun eenvoudige morfologie. Latere biochemische analyses wezen uit, dat microbodies waren onder te verdelen in een aantal onderling zeer verschillende groepen organellen. Tegenwoordig worden microbodies op grond van deze biochemische eigenschappen onderverdeeld in peroxisomen, glycosomen, hydrogenosomen en glyoxysomen. Deze studie is gericht op het peroxysoom. In dit celcompartiment vindt een grote diversiteit aan metabole activiteiten plaats. Het type cel en de fysiologische condities waarin deze cel verkeert, bepalen de grootte, vorm en het aantal van de peroxisomen in deze cel. Tevens hebben deze factoren een grote invloed op het type metabole processen die zich in het peroxysoom afspelen. Wanneer de metabole behoeften veranderen, kan ook de aanwezigheid van peroxisomen hierop worden aangepast. Dit maakt het peroxysoom tot een veelzijdig en flexibel organel. Het grote belang van peroxisomen in het metabolisme van een gezonde cel wordt waarschijnlijk het best geïllustreerd door het voorkomen van enkele zeer ernstige, soms zelfs dodelijke, aandoeningen bij de mens die worden veroorzaakt door afwijkingen in het functioneren van peroxisomen. De verzamelnaam voor deze aandoeningen is **PBD** (**P**eroxisome **B**iogenesis **D**isorders).

Gisten zijn zeer geschikte organismen voor het bestuderen van de vorming (biogenese) van peroxisomen. Mutanten van deze organismen, waarin dit proces verstoord is (zogenaamde ***pex*** mutanten) zijn namelijk levensvatbaar, mits gekweekt onder de juiste condities. Dit maakt het mogelijk om het defecte gen en het bijbehorende eiwitproduct te identificeren. Op deze wijze zijn al 25 eiwitten die direct

betrokken zijn bij peroxysoom biogenese, de zogenaamde “peroxins”, ontdekt. Gisten zijn bij uitstek geschikt als modelorganismen voor de studie naar de oorzaken van PBD’s. Op basis van informatie uit het onderzoek naar peroxysoom biogenese in gisten, zijn van 11 van de 12 nu bekende PBD’s de oorzaken op moleculair niveau bekend.

Bij onderzoek naar de biogenese van peroxisomen is jarenlang uitgegaan van het “growth and fission” (“groei en deling”) model als verklaring voor de wijze van vermeerdering van deze organellen. Dit model stelt, dat nieuwe peroxisomen worden gevormd uit reeds bestaande organellen, door een proces van groei gevolgd door deling. In geval van zich delende cellen zijn de peroxisomen in de dochtercel via deling elk afkomstig van de moedercel. Dit model sluit de vorming van nieuwe peroxisomen *de novo* (“uit het niets”) uit. Diverse recente onderzoeksresultaten geven aanleiding om aan het groei en deling model te twijfelen. Deze resultaten lijken erop te wijzen dat in ieder geval in een aantal organismen de mogelijkheid van een alternatieve herkomst van peroxisomen niet kan worden uitgesloten. De in dit proefschrift beschreven studie was erop gericht de mogelijkheid van peroxysoom biogenese anders dan volgens het “groei en deling” model, aan een nader onderzoek te onderwerpen. In deze studie is gewerkt met de methylotrofe (“op methanol groeiende”) gist *Hansenula polymorpha*. Voor groei op methanol is de aanwezigheid van intakte peroxisomen essentieel: *pex* mutanten van *H. polymorpha* groeien dus niet op methanol.

Hoofdstuk 1 geeft een overzicht van onze huidige kennis van peroxysoombiogenese en beschrijft de rol van dit organel in de cel. Ook wordt hierin een overzicht gegeven van in de literatuur beschreven onderzoeksresultaten die wijzen op het bestaan van alternatieven op het groei en deling model voor peroxysoom vorming.

In hoofdstuk 2 wordt de isolatie van het *PUR7* gen beschreven. Dit gen is gevonden door te zoeken naar dàt stuk van het DNA (genoom) van de gist *H. polymorpha* dat in staat was om het specifieke defect van de *ade11.1* mutant te herstellen (complementeren). Deze mutant kan geen purines (één van de bouwstenen van DNA) synthetiseren en is daarom niet in staat te groeien op medium zonder adenine (een purine). Het gevonden stuk DNA bleek na verdere analyse het gen te bevatten dat codeert voor het eiwit betrokken bij de zevende stap van de purine biosynthese (*PUR7*). Door het *PUR7* gen te kloneren in een plasmide (een circulair stuk DNA dat onafhankelijk van het genoom door de cel wordt vermeerderd) is een belangrijk stuk genetisch gereedschap voor het werken met *H. polymorpha* gemaakt. Dit plasmide, pHIPA4, is namelijk geschikt gebleken om op efficiënte wijze eiwitten te laten

overproduceren in de *ade11.1* mutant. Dit is aangetoond met behulp van de modeleiwitten amine oxidase en catalase, beide peroxysomale eiwitten. Het gebruik van de *ade11.1* mutatie, vergroot de mogelijkheden voor genetisch modificeren van *H. polymorpha*. De toepasbaarheid van dit organisme in fundamentele en toegepaste studies is hiermee vergroot.

Het Pex3 eiwit (Pex3p) is reeds enige jaren geleden geïdentificeerd als peroxin. Het *PEX3* gen bleek de *pex3* mutant te complementeren. Karakteristiek voor de *pex3* mutant is de totale afwezigheid van peroxysomale resten ("ghosts"). Een meerderheid van de bekende *pex* mutanten bezit dergelijke ghosts wél, wat erop duidt dat zij in staat zijn tot een min of meer normale vorming (assemblage) van de peroxysomale membraan. De afwezigheid van ghosts in de *pex3* mutant duidt op een belangrijke rol van Pex3p in de synthese van de peroxysomale membraan. Daarom is Pex3p een belangrijk instrument om meer inzicht te krijgen in peroxysoombiogenese. Hoofdstuk 3 beschrijft een studie naar de lokalisatie van Pex3p. Er is gebruik gemaakt van vier varianten van Pex3p, die op verschillende plaatsen een specifieke, herkenbare aminozuursequentie bevatten. Deze aminozuurvolgorde, de zogenaamde myc-tag (EQKLISEEDL), kon vervolgens worden gebruikt bij de detectie van deze eiwitten. Eerst werd de functionaliteit van de varianten van Pex3p getest door de complementatie van de *pex3* mutant te controleren. Hieruit bleek dat alle Pex3p-myc constructen gelijk aan Pex3p functioneren en dus het vermogen van de mutant om te groeien op methanol herstellen. Via toepassing van verschillende technieken is vervolgens de oriëntatie van Pex3p in de peroxysomale membraan onderzocht. Door het toevoegen van een protease (een eiwit dat andere eiwitten in stukken knipt) aan intacte peroxisomen werd aangetoond dat alle myc-tags gevoelig waren voor de werking het protease. In een andere serie experimenten werd door het toevoegen van specifieke anti-myc antilichamen (eiwitten die met hoge affiniteit binden aan myc-tags) de aanwezigheid van de tags aan de buitenzijde van peroxisomen vastgesteld. Tenslotte werd de wijze waarop het eiwit aan de peroxysomale membraan bevestigd is onderzocht door middel van een extractie experiment. Uit dit laatste experiment bleek dat Pex3p zeer sterk gebonden is aan de membraan. Gezamenlijk wijzen deze experimenten erop dat Pex3p een zeer hechte interactie aangaat met de buitenzijde van het peroxysoom, zonder daarbij in de membraan aanwezig te zijn met "membraanspannende segmenten". Dit resultaat is duidelijk anders dan hetgeen al in de literatuur beschreven was.

Waarschijnlijk bindt Pex3p aan een ander eiwit in de peroxysomale membraan. Tot op heden is niet bekend welk eiwit deze bindingspartner is. Verder bewijs voor deze

lokalisatie van Pex3p werd gevonden met behulp van vriesets elektronenmicroscopie. Met deze methode werd vastgesteld, dat overproductie van Pex3p een totaal ander beeld van de membraan opleverde dan bij overproductie van eiwitten met meerdere membraanspannende segmenten. Wanneer cellen die een fusie van Pex3p aan GFP (een fluorescent merkereiwit) produceren werden bestudeerd met fluorescentie microscopie, leverde dit een onverwacht resultaat op. De fluorescentie bleek namelijk aanwezig te zijn in zeer specifieke locaties op de membraan. Deze concentratie in “hot spots” lijkt erop te duiden dat Pex3p zich op duidelijk gedefinieerde plaatsen in de peroxysomale membraan bevindt.

Welke gebeurtenissen vinden plaats wanneer in *pex3* cellen een deel van Pex3p wordt geproduceerd? Tot onze verrassing bleek een mutant ($Pex3p_{[1-50]}$), die een deel van het begin (N-terminus) van Pex3p ter grootte van 50 aminozuren bevatte, grote aantallen vesicles (blaasjes) te bevatten. In hoofdstuk 4 wordt de herkomst en aard van deze vesicles in detail beschreven. De blaasjes bleken binnen 30 minuten na begin van de productie van $Pex3p_{[1-50]}$ in de *pex3* mutant te worden gevormd in de nabijheid van de kernmembraan. Zowel biochemisch als door middel van elektronenmicroscopie kon worden aangetoond dat de vesicles naast het $Pex3p_{[1-50]}$ eiwit ook Pex14p bevatten, een peroxysomaal membraaneiwit. Ook alcohol oxidase, een peroxysomaal matrixeiwit, werd teruggevonden in de vesicles. Dit betekent, dat deze vesicles peroxysomale eigenschappen bezitten, maar geen echte peroxisomen zijn. Het deel van Pex3p dat ontbreekt in het $Pex3p_{[1-50]}$ eiwit, de aminozuren 51 tot 457, is blijkbaar nodig om normale peroxisomen te vormen. Wanneer vervolgens het volledige Pex3p werd geproduceerd in cellen waarin zich eerst vesicles hadden gevormd, bleken inderdaad nieuwe peroxisomen te ontstaan tussen de blaasjes. Nadere analyse wees uit, dat deze peroxisomen gevormd werden uit een aantal van de vesicles. Dit kon worden bewezen doordat de nieuwe organellen het merkereiwit GFP, waarmee de vesicles gemerkt waren, bevatten. Dit toont aan dat structuren afkomstig van de kernmembraan direct kunnen worden gebruikt bij de vorming van nieuwe peroxisomen onder deze condities. Dit is een voorbeeld van peroxysoombiogenese buiten het klassieke groei en deling model om. De vesicles kunnen worden beschouwd als een tussenstadium in het proces van peroxysoombiogenese vanuit de kernmembraan.

Tenslotte wordt in hoofdstuk 5 beschreven wat er gebeurt in *pex3* cellen wanneer het volledige Pex3p wordt teruggebracht. In deze situatie blijkt zich een enkel organel te vormen in directe nabijheid van de kernmembraan. Door gebruik te maken van een eiwit dat zich ophoopt in het ER (en dus ook in de kernmembraan) BiP-GFP, wordt aangetoond dat het peroxysoom naar alle waarschijnlijkheid wordt gevormd uit een

deel van het ER, omdat GFP bij nadere analyse terug te vinden is in de nieuw gevormde organellen. Samen met de gegevens beschreven in hoofdstuk 4, duidt dit erop dat peroxisomen wel degelijk kunnen ontstaan uit het ER, en niet uitsluitend uit andere peroxisomen.

In het recente verleden zijn aanwijzingen voor de mogelijkheid van peroxysoom biogenese in afwezigheid van reeds aanwezige peroxisomen gevonden. Meerdere onderzoeksgroepen hebben hieraan hun bijdrage geleverd. Ook de in dit proefschrift beschreven resultaten duiden op een biogenese route die afwijkt van het klassieke model. Welke rol deze route speelt in normale, “wildtype”, *H. polymorpha* cellen is nog onduidelijk. Mogelijk vindt deze wijze van peroxysoomvorming nooit plaats in wildtype cellen, maar is zij uitsluitend een herstelmechanisme dat optreedt wanneer de organellen verloren gaan, bijvoorbeeld tijdens celdeling. Nader onderzoek zal moeten plaatsvinden om openstaande vraagstukken, zoals de identiteit van het eiwit dat misschien betrokken is bij het herkennen van Pex3p naar het kernmembraan, te beantwoorden.

List of abbreviations

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]1-propane-sulfonic acid
ER	endoplasmic reticulum
GFP	green fluorescent protein
MES	4-morpholineethanesulfonic acid
PNS	postnuclear supernatant
WT	wild type
ADH	alcohol dehydrogenase